Cell Type-Specific Regulation of the Chicken Tyrosinase Promoter

by

Ruth Elsie Clarke

Thesis presented for the degree of
Master of Science (Medicine)
in the Department of Anatomy and Cell Biology in the
Faculty of Medicine
University of Cape Town

July 2000

DECLARATION

I, Ruth Elsie Clarke, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for any other degree in this or any other University. I empower the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signed by candidate

.....

July, 2000

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Prof. Sue Kidson: for patiently nurturing me, for chiding me when I needed it, for constant encouragement and never failing to be excited about each little triumph along the way. Above all, thank you, Sue, for challenging me to think beyond my own research area - for always being inspired and inspiring about science!

I would like to thank all my colleagues in the lab for many hours of help and friendship. Thanks also to Prof. Iqbal Parker from the Department of Medical Biochemistry for helpful discussions.

To both my families: thank you, every one of you, for your unfailing support, for asking questions and listening to my answers, for being interested! A special word of thanks to my mum and dad for exposing me to the wonder of nature from my earliest years, and for always encouraging me to pursue my interest in science.

To Trent, without whom none of this would have been possible: thank you for setting me free to follow my heart. And mostly, thank you for cheering me on every step of the way. Now onto new adventures!

Finally, thanks to my Creator, maker of heaven and earth: *"I praise you because I am fearfully and wonderfully made. All your works are wonderful, I know that full well'. Psalm 139:14*

ABSTRACT: Cell type-specific regulation of the chicken tyrosinase promoter.

Melanin, the pigment found in the eyes and coats of vertebrates, is synthesised by two main cell types: melanocytes and retinal pigment epithelial (RPE) cells. These two cell populations, which arise from distinct embryological origins, differ with respect to the rate at which they produce melanin and the ways in which they respond to melanogenic stimuli. Tyrosinase is the rate-limiting enzyme in the melanin synthesis pathway, and the regulation of tyrosinase gene expression in mammalian melanocytes has been extensively studied. In contrast, regulation of tyrosinase gene expression in RPE cells has received little attention. In the present study, the chicken tyrosinase gene promoter was used to investigate possible differences in the regulation of tyrosinase expression in melanocytes and RPE cells. Transient transfection experiments were carried out in which reporter constructs, consisting of tyrosinase promoter deletion fragments linked to a luciferase reporter gene, were introduced into melanocytes, RPE cells and a non-pigmented cell line. The following results were obtained. (1) Reporter expression obtained with the longest (2.1kb) promoter fragment was significantly higher in pigmented cells (both melanocytes and RPE cells) than in non-pigmented cells, demonstrating the pigment cell-specificity of the chicken tyrosinase promoter. (2) Reporter expression obtained with a 0.5kb promoter fragment, containing conserved core regulatory elements (an Inr, M-box and Sp1 binding site), was higher in melanocytes than in RPE cells. This result suggests that the core elements are sufficient for high levels of tyrosinase expression in melanocytes, but not in RPE cells. (3) Reporter activity obtained with a 248bp promoter fragment containing no elements implicated in initiating tyrosinase transcription was strikingly high in RPE cells, and very low in melanocytes. This result suggested the presence of RPE-specific regulatory elements in the tyrosinase promoter. To determine which portion of the 248bp promoter fragment contained the element(s) responsible for this RPE-specific activity, three additional deletion constructs were cloned. Transient transfection experiments with these new constructs revealed that the RPE-effect observed with the 248bp construct was a serendipitous / unfortunate experimental artefact brought about by the ligation of 203bp of proximal promoter with 45bp of distal promoter. Examination of the sequence generated by this ligation revealed the presence of an element similar to PCE-1, an element recently implicated in RPE-specific gene regulation. Factors present in RPE cells, but not in melanocytes, may bind to this element to initiate transcription. Further investigation of the mechanism mediating this RPE-specific effect could contribute to the understanding of RPE-specific gene regulation. In conclusion, the results of the present study strongly suggest that expression of the chicken tyrosinase gene is regulated differently in RPE cells and melanocytes, and begin to identify regions in the chicken tyrosinase promoter that might be responsible for mediating such differences.

TABLE OF CONTENTS

CHAPTER ONE: General introduction and aims

INTRODUCTION

The pigment melanin is found in the eyes and coats of vertebrates, and in a few other minor locations such as the inner ear and regions of the brain. Melanin is synthesised in two types of pigment producing-cells, namely, melanocytes and retinal pigment epithelial (RPE) cells. Melanocytes are found in skin, hair follicles and feather buds and in the uvea (choroid and iris) of the eye, while RPE cells form the specialised monolayer that lies beneath the neural retina. Although they both synthesise melanin, these two cell types have different embryological origins. In addition, the synthesis of pigment in the two cell populations differs in certain aspects, for example, with respect to their response to melanogenic stimuli and the rate at which the cells synthesise pigment. Many studies have investigated the mechanisms whereby melanogenesis is regulated in melanocytes. However, very little is known about the regulation of melanogenesis in RPE cells and how this differs from melanogenic regulation in melanocytes.

Tyrosinase is a key enzyme in the biochemical pathway that results in the formation of melanin from the amino acid substrate, tyrosine. For this reason, it is a useful tool for investigating the biological control of pigmentation. In the last ten years, the regulation of tyrosinase gene activity has been extensively researched in mammals. However, the regulatory region of the chick tyrosinase gene was isolated only fairly recently, and relatively little is known about how this promoter functions. The purpose of this study therefore, is to further our understanding of how melanogenesis is regulated differently in melanocytes and RPE cells, by making use of chicken tyrosinase promoter constructs in cultured melanocytes and RPE cells.

SECTION A: Introduction to pigment cell biology

1.1 Melanin and melanosomes

Melanin pigments are found in the eyes and skin, hair or feathers of vertebrates. The two major types of melanins are the eumelanins (brown and black pigments) and the phaeomelanins (red and yellow pigments). Melanins serve diverse functions, from camouflage and protection against the

harmful effects of ultra-violet radiation, to absorption of scattered light in the eye and free-radical scavenging (reviewed by Hill et al, 1997). Melanins are also found in a small population of melanocytes in the ear, where their importance in hearing is acknowledged but not understood, and as neuromelanins in specific regions of the brain, where their functional significance has become clearer through studies of neurodegenerative disorders such as Parkinson's disease (Tief et al, 1998).

In pigment cells, melanin is synthesised in specialised organelles known as melanosomes. Melanosomes contain an inner matrix that acts as a scaffold on which melanin is laid down. Melanosomes arise from the endoplasmic reticulum and go through several maturation stages before they become visibly pigmented as a result of their ultimate saturation with melanin pigments. They are thought to share the same origin as lysosomes and to be closely related to these organelles (Zhou et al, 1993).

1.2 Cell biology of melanocytes and RPE cells

Melanocytes of the skin are stellate cells situated in the basal layer of the epidermis, with each melanocyte surrounded by a number of keratinocytes (Fig 1.1). Fully pigmented melanosomes travel down the cytoplasmic extensions of the melanocyte and are then transferred to the surrounding keratinocytes (in humans) or to the hairs or feathers of other animals. The primary function of this coat melanisation seems to be to confer protection to the cells from the harmful effects of ultraviolet radiation, perhaps by scattering incoming light. In addition, melanins have been shown to scavenge damaging free-radicals and active oxygen species formed when sunlight impinges on the skin, thereby preventing UV-induced DNA damage. However, there is evidence to suggest that melanin is also a photosensitiser and that melanins may actually enhance DNA damage (Hill et al, 1997). In addition to their obvious melanin-related functions, a number of non-pigmentary functions have been identified for melanocytes. These include roles in tissue repair, immune and inflammatory responses and roles for several of the colourless intermediaries of the melanogenic pathway (Prota, 1997).

The retinal pigment epithelium is one of the most important cell layers in the visual system, as it supports the development and functioning of the neural retina in a number of essential ways (Raymond and Jackson, 1995). Without the RPE, the integrity of the neural retina cannot be maintained. Viewed face-on, a sheet of RPE presents a "cobblestone" mosaic of polygonal cells.

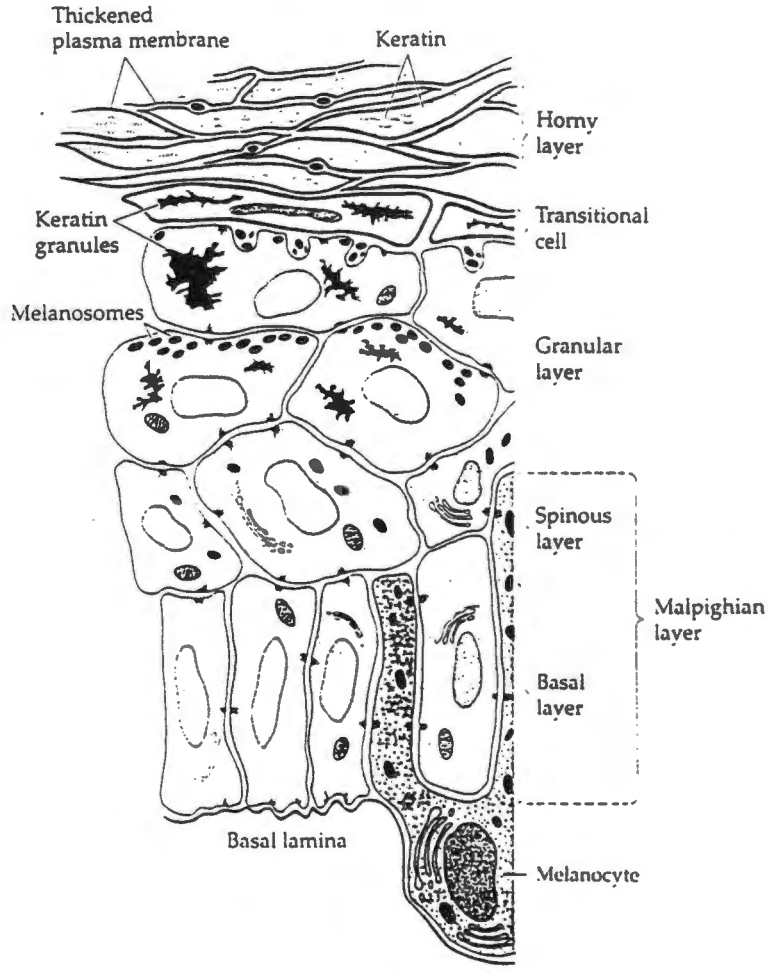


Fig 1.1: The position and role of melanocytes in the epidermis. The layers of the epidermis are shown. The basal epidermal cells are mitotically active whereas fully keratinised cells characteristic of external skin are shed off. The keratinocytes obtain pigment from the transfer of melanosomes from the melanocytes that reside in the basal layer of the epidermis. After Gilbert, 1994.

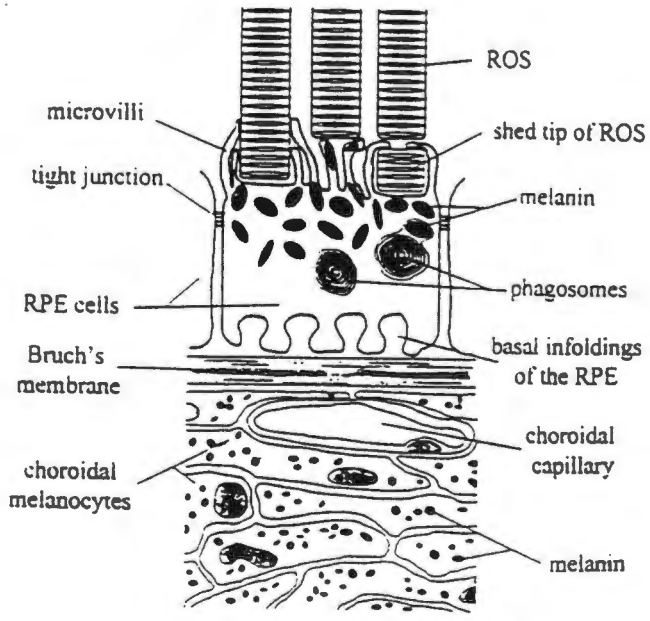


Fig 1.2: The position and structure of RPE cells in the retina. The RPE rests on Bruch's membrane, between the vascular choroid and the sensory retina. Apical specialisations of RPE cells include cylindrical microvilli investing the tips of the photoreceptor outer segments, and apical melanosomes. After Schraermeyer and Heimann, 1999.

The cells are roughly cuboidal, polarised with respect to their apical-to-basal axis (Fig 1.2). Melanosomes synthesised in RPE cells are transported into apical microvilli where they absorb scattered light and block light coming through the sclera. This prevents degradation of the visual image and provides protection from light damage to the photoreceptors. However, pigmentation is just one of many ways in which the RPE serves the neural retina. For example, tight junctional complexes at the apical side of the lateral cell membranes form a permeability barrier, part of the **blood-retina barrier**, which prevents the entrance of toxic levels of serum components to the outer retina. The RPE provides **physical protection** to the rods and cones by means of unusual apical microvilli of RPE cells that are modified to form cylindrical sheaths investing the tips of the photoreceptor outer segments. In addition, **phagocytosis** of shed rod and cone outer segment discs occurs at the microvilli. The RPE also plays a role in the **transport** of many substances to and from the photoreceptors. In particular, the RPE is involved in the metabolism of the vitamin A-derived pigments of the visual cycle (for reviews, see Zhao et al, 1997; Schraermeyer and Heimann, 1999).

1.3 Important differences between melanocytes and RPE cells

Developmental origins of melanocytes and RPE cells

Apart from their ability to produce melanin, RPE cells and melanocytes of the skin have little in common. It is probable that many of the important differences in melanogenesis in melanocytes and RPE cells arise from their different developmental origins. Melanocytes are derived from the neural crest, a transient aggregation of multipotent cells that migrate out of the closing neural tube during neurulation of the developing embryo (Fig 1.3). Cells migrating from the neural crest follow well-characterised migration pathways to diverse locations in the body where they give rise to a variety of cell types. In mammals and avians, cells destined to become melanocytes migrate along the dorsolateral pathway to eventually populate the epidermis, where they may become localised in hair follicles or feather buds (Fig 1.4). Neural crest cells also migrate to the developing eye, where they give rise to the melanocytes of the uvea (amongst other things). Although intensively studied, it has still not been established at which stage neural crest cells lose their pluripotency and become melanoblasts, committed to the melanocyte lineage. Once differentiated, melanocytes of mammalian hair follicles continue to proliferate. They are apparently derived anew from unpigmented precursors with each cycle of hair growth (Protten and Chase, 1970, in Porter et al, 1999).

In contrast to melanocytes, the retinal pigment epithelium is derived from the neuroepithelium of the optic cup. The vertebrate eye develops as an evagination, called the optic vesicle, of the lateral wall of the developing forebrain. As it grows towards the surface of the head, the distal end of the vesicle enlarges while the connection with the brain narrows to form the optic stalk, the site of the future optic nerve. As it approaches the surface ectoderm, the optic vesicle invaginates to form a double-walled optic cup. The inner wall of the cup gives rise to all the cellular layers of the neural retina, while the outer layer eventually gives rise to the retinal pigment epithelium (Fig 1.5). Interestingly, experiments have shown that initially, each layer of the optic cup can give rise to both neural retina and RPE, depending on the signals received from adjacent tissues. Much work has been done in an attempt to elucidate the mechanisms whereby the fate – RPE or neural retina – of these two initially identical cell layers is specified and restricted. In contrast to melanocytes, retinal pigment cells are post-mitotic in adult life and exist as senescent (rather than simply quiescent) cells, with concomitant phenotypic changes (Hjelmeland, 1999).

Implications of different developmental origins for the regulation of melanogenesis in melanocytes and RPE cells

Because they have different developmental origins, melanocytes and RPE cells reach their differentiated, pigment-producing states via completely different routes. Melanocytes, for example, which originate from migratory precursor cells, receive developmental signals from the mesodermal cells that line their migratory pathway. RPE cells are non-migratory, and must receive their developmental signals from the overlying ectoderm or the underlying neuroepithelium and mesoderm. Thus, it seems highly likely that different cascades of regulatory genes must be involved in the development and differentiation of these two cell types.

For example, microphthalmia (microphthalmia-associated transcription factor / Mitf) is a transcription factor that has been shown to have essential roles in melanocyte development. In particular, Mitf has been shown to transactivate tyrosinase gene expression, via binding to E-box motifs found in the tyrosinase gene promoter (see section 1.5 for further details) (for reviews, see Moore, 1995; Jackson and Raymond, 1994). Thus, *Mitf* is expressed upstream of tyrosinase in both melanocytes and RPE cells. Recently, the 5' flanking regions of the human *MITF* gene have been cloned and characterised (Fuse et al, 1996; Udono et al, 2000). This has allowed Watanabe et al (1998) to demonstrate, using co-transfection experiments, that Pax3, a transcription factor with a paired domain and a homeodomain, is able to transactivate the *Mitf* promoter. So, Pax3 is expressed upstream of *Mitf*. Pax3 is expressed in melanocytes. Most interestingly, however, Pax3 is not expressed in RPE cells and it is not yet known what switches *Mitf* on in the RPE. Therefore,

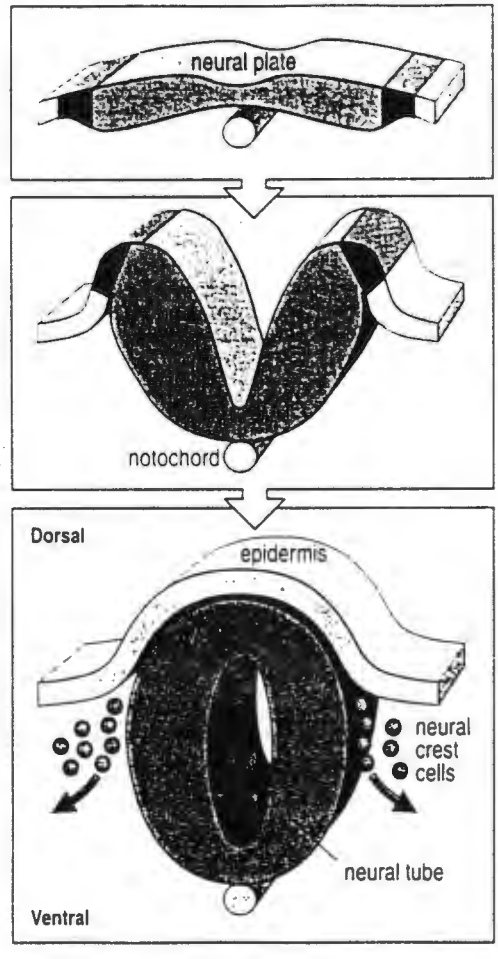


Fig 1.3: Formation of the neural crest, a transient aggregation of multipotent cells that migrate out of the closing neural tube during neurulation of the developing embryo. After Wolpert et al, 1998.

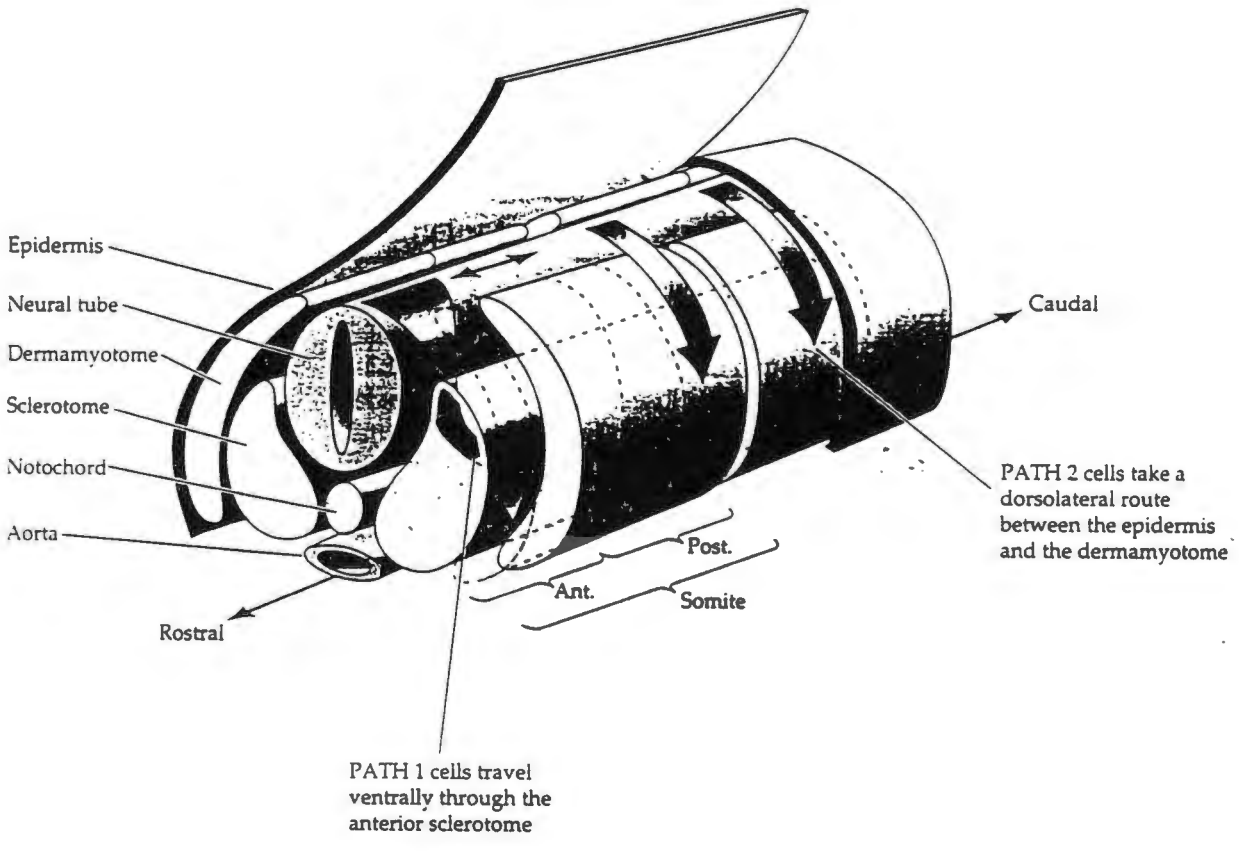


Fig 1.4: Neural crest cell migration in the trunk of the chick embryo. Path 1: Cells travel ventrally through the anterior of the sclerotome (the portion of the somite that generates vertebral cartilage). These cells contribute to the sympathetic and parasympathetic ganglia as well as to the adrenal medullary cells and dorsal root ganglia. Path 2: Somewhat later, cells enter a dorsolateral route beneath the ectoderm. These cells become pigment-producing melanocytes. After Gilbert, 1994.

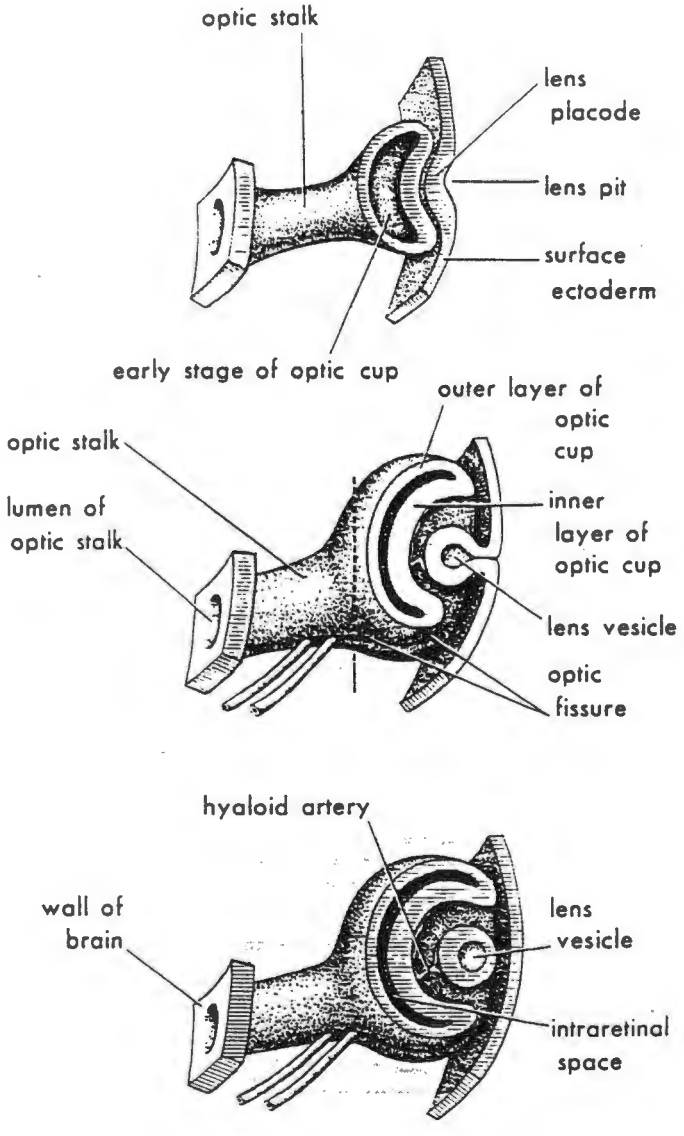


Fig 1.5: Development of the eye cup and the RPE. The eye cup develops as an evagination of the developing forebrain. The eye cup invaginates, giving rise to inner and outer cellular layers. The RPE develops from the outer layer of the optic cup. After Moore, 1977.

the following important point can be made: the gene cascade leading to tyrosinase expression in the RPE is likely to be quite different to that in melanocytes.

Regardless of different upstream signalling events, at some stage melanocytes and RPE cells must activate a common set of genes in order for melanogenesis to take place. The question arises: at what stage of pigment cell differentiation do different factors regulate melanogenesis in melanocytes and RPE cells? There is evidence, concerning the **rate** of tyrosinase transcription in RPE cells and melanocytes and the **response** of the tyrosinase promoter to extracellular signals, to suggest that apart from upstream differences in the cascade, at least some differences occur at the level of tyrosinase transcription. This aspect of the regulation of melanogenesis is most relevant to the present study, and is discussed below.

Rate of tyrosinase transcription in melanocytes and RPE cells

Skin melanocytes transfer mature melanin granules into the surrounding keratinocytes, while RPE cells retain their granules, perhaps throughout life. This observation raises the question of how melanin is turned over in RPE cells versus melanocytes, which in turn has direct implications for the understanding of the mechanisms controlling tyrosinase activity in the two cell types. Does melanin production in RPE cells cease once the cell is saturated with mature melanin granules? The limited amount of available evidence seems to indicate that tyrosinase activity in the RPE first rises and then falls with increasing developmental age, and that pigmentation of the RPE is completed well before birth. Thus, it is held that because tyrosinase activity is no longer required postnatally, tyrosinase expression is essentially down-regulated after birth. Postnatal darkening of the eyes documented in both mice and humans (Pierro, 1963; Matheny and Dolan, 1975) is therefore attributed exclusively to postnatal melanogenesis in the neural crest-derived melanocytes of the iris stroma (Chiu et al, 1993). The issue is far from settled, however, in part because there is evidence for the continuation of melanogenesis in the RPE throughout life (reviewed by Schraemeyer and Heimann, 1999). Either way, it is likely that the postnatal rate of tyrosinase transcription in RPE cells is significantly lower than in skin melanocytes.

Melanogenic response of melanocytes and RPE cells to extracellular stimuli

It is well known that one of the ways in which melanocytes respond to a number of extracellular stimuli, such as ultraviolet (UV) radiation and α-melanocyte stimulating hormone (α-MSH), is by upregulating tyrosinase expression. The mechanisms whereby these agents regulate melanogenesis have been extensively studied in melanocytes and melanoma cells (for review, see Ferguson and Kidson, 1997). In contrast, little is known about whether RPE cells are responsive to the same

stimuli. For example, it has been shown that melanocytes in the skin respond to UV irradiation by increasing melanin synthesis and increased proliferation (reviewed by Ferguson and Kidson, 1997). However, it is not known whether RPE cells likewise upregulate tyrosinase expression in response to UV radiation. This is a particularly interesting question because RPE cells, by virtue of their location in the eye, are continually exposed to high levels of UV radiation. These questions remain to be answered.

There are sufficient differences in the development and physiology of RPE cells and melanocytes to suggest that tyrosinase must be regulated differently in these cell types. In the present study, a functional analysis of the chicken tyrosinase promoter was carried out with the specific aim of searching for cis-acting promoter elements that might be responsible for regulating tyrosinase expression differently in melanocytes and RPE cells. For this reason, it is necessary to review what is currently known about the regulatory elements of the tyrosinase promoter.

SECTION B: The regulation of tyrosinase gene expression.

As mentioned previously, tyrosinase is the first and rate-limiting enzyme in the melanogenic pathway. In the absence of tyrosinase, no melanin is produced and albinism results. Tyrosinase (Tyr) initiates the process of melanin synthesis by converting tyrosine to dihydroxyphenylalanine (DOPA), and catalyses two further steps in the pathway (Fig 1.6). Two additional enzymes involved in the pathway are the Tyrosinase-related protein-1 (Trp-1), and Dopachrome tautomerase (DCT, previously called Tyrosinase-related protein-2 or Trp-2) (Del Marmol and Beermann, 1996). Together these three enzymes, which share an amino acid homology of 40% and have a number of structural features in common (reviewed by Jackson et al, 1994), comprise the tyrosinase gene family.

Given its pre-eminent role in the melanogenic pathway, one of the obvious mechanisms for control of melanogenesis is regulation of tyrosinase gene activity. Such regulation can occur at different levels. For example, pre-exisisting pools of inactive tyrosinase enzyme may be activated at a post-translational level by glycosylation of the enzyme (Park et al, 1993). Alternately, many factors known to regulate melanogenesis do so by acting, either directly or indirectly, at the level of tyrosinase gene transcription. That is, control of melanogenesis is mediated via mechanisms that cause a change in tyrosinase mRNA levels. For the purposes of this review, only regulation of tyrosinase gene transcription will be discussed.

In the following section, a brief overview of the structure of the tyrosinase gene promoter is presented. This is followed by a more detailed discussion of the functional significance of various promoter elements in mediating transcription initiation, cell type-specific expression, and wild-type levels of tyrosinase gene expression. Attention is then given to a review of mechanisms that may allow tyrosinase expression to be regulated differently in melanocytes and RPE cells. A further area of regulation that is not discussed in this review, involves the modulation of tyrosinase expression in response to humoral stimuli, such as ultraviolet light and α-MSH, known to influence melanogenesis (for review, see Ferguson and Kidson, 1997). Because the present study was carried out using both mammalian and avian cell lines, the review that follows includes information gleaned from studies of both the avian and mammalian tyrosinase promoters.

1.4 Comparative overview of the arrangement of the mammalian and avian tyrosinase gene promoters

Genomic clones for the tyrosinase genes of a number of vertebrates, including human, mouse, chicken and quail, have been isolated (Kikuchi et al, 1989; Yamamoto et al, 1989; Ferguson and Kidson, 1996; Yamamoto et al, 1992). Several cis-acting promoter elements that allow for initiation and modulation of tyrosinase gene expression have been identified in functional analyses of the 5' regulatory regions of the mammalian genes. Similar characteristic sequences have been identified in the promoter regions of the avian (chicken and quail) tyrosinase genes. However, the functional significance of these sequences in avians is poorly understood. In addition to regulatory elements in the 5' flanking regions of the tyrosinase promoter, a recent report has identified a sequence in the 3' non-coding RNA fragments of the mouse tyrosinase gene that may be involved in feedback regulation of tyrosinase expression (Takeuchi et al, 2000).

Alignment of the avian, human and mouse tyrosinase promoter sequences has revealed two conserved regions: a proximal conserved region and a distal conserved region (Ferguson and Kidson, 1996). These regions contain regulatory elements, including an initiator region (Inr), reported to play a role in pigment cell-specific expression of the tyrosinase gene in mammals. Interestingly, the homology between the mammalian and the avian promoters ends abruptly just downstream of the Inr (see Fig 1.7). Indeed, the chicken and quail promoters have approximately 100bp between the Inr and the translation start point (ATG), that are not found in the mammalian promoters (Ferguson and Kidson, 1996). The possible functional significance of this 'extra' region

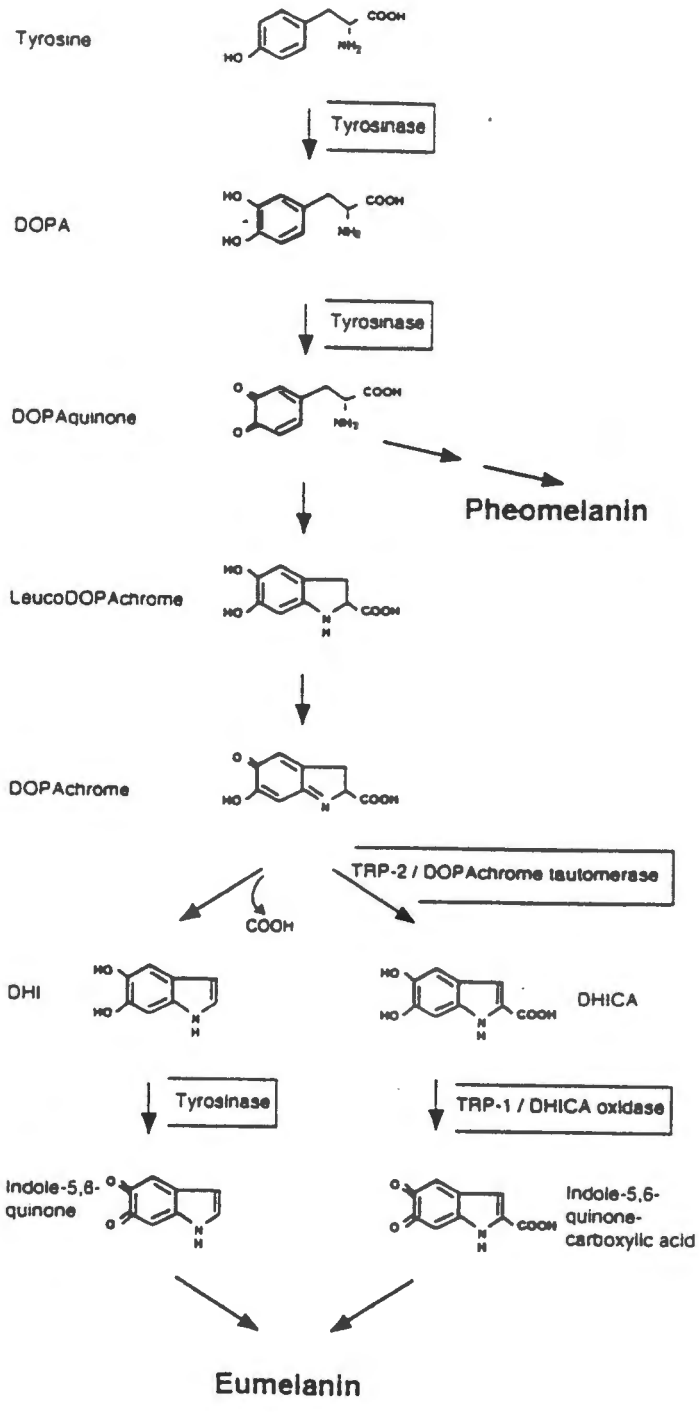


Fig 1.6: The melanin biosynthesis pathway. Enzymatic reactions attributed to tyrosinase, Trp1 and Trp2 (DCT) are indicated. DOPA: 3,4-dihydroxy-phenylalanine; DHICA: 5,6-dihydroxy-indole-2-carboxylic acid; DHI: 5,6-dihydroxy-indole. After del Marmol and Beermann, 1996.

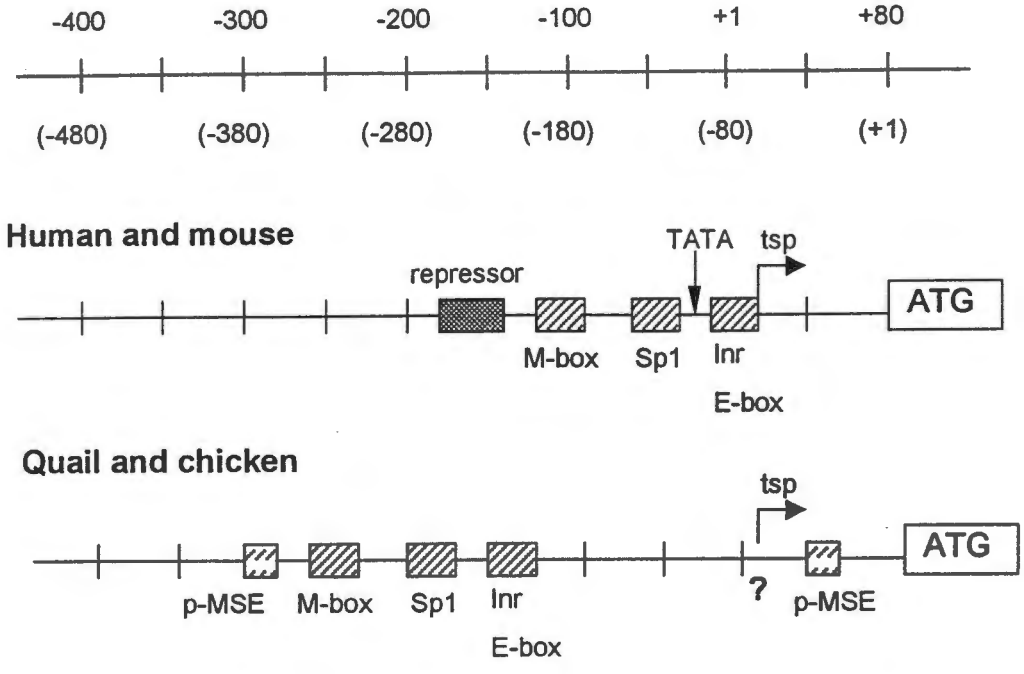


Fig1.7: Relative positions of regulatory elements identified in the proximal promoter regions of the mammalian (human and mouse) and avian (quail and chicken) tyrosinase promoters. Adapted from Ferguson and Kidson (1997).

Transcription start point (tsp): the major transcription start point for the mammalian genes has been fairly accurately mapped. In avians however, a predominant site has not yet been singled out.

ATG: the position of the translation start codon is indicated with this symbol.

TATA boxes: TATA-like elements are found in the mammalian tyrosinase promoters but not in the avian gene.

Initiator region: an initiator (Inr) region, containing a conserved E-box motif, is found in all the promoters.

Sp1 binding site: a non-consensus binding site was identified in the human promoter, and similar sequences are found in the mouse and avian promoters.

M-box: this highly conserved motif is implicated in the pigment cell-specific expression of the tyrosinase gene. Additional M-box-like elements in the avian promoters have been termed **p-MSE.**

is not yet known, although the results of pilot experiments (presented later in this section) have shed some light on this question.

An overview of the arrangement of elements in the proximal tyrosinase promoter regions of the human, mouse and avian (chicken and quail) tyrosinase genes is presented in Fig 1.7. The functional significance of these elements is discussed in some detail in the following paragraphs, and the reader is referred to Fig 1.7 throughout.

1.5 What elements are required for basal initiation of tyrosinase gene transcription?

Initial advances in understanding the basic mechanisms of transcription initiation by RNA polymerase II were made in studies of promoters whose activities depend on a common control element named the TATA box. It is well established that this element functions to determine efficient transcription initiation 25 to 30bp downstream by binding the multiprotein complex TFIID. Although both the human and mouse tyrosinase promoters have TATA-like sequences approximately 32bp upstream of the transcription start site at –80, neither promoter has a canonical TATA motif. Several TATA motifs have been identified in the chicken tyrosinase promoter but the most proximal of these is at –552bp from the ATG and cannot be functioning to initiate transcription (Ferguson and Kidson, 1996).

It has now become clear that many promoters do not contain consensus or even non-consensus TATA boxes. In the absence of a TATA box, an element called an initiator (Inr), acts as a functional analogue of the TATA box, directing accurate transcription initiation by RNA polymerase II (for review, see Smale, 1997). Such an initiator sequence is found in the human, mouse, quail and chicken promoters (Bentley et al, 1994; Ferguson and Kidson, 1996). The initiator overlaps the major transcription start site for the human and mouse tyrosinase gene, but is found slightly further upstream in the avian promoters (Fig 1.7). It consists of an overlapping E-box motif (CATGTG) and a non-consensus octamer element, GTGATAAT (Bentley et al, 1994). The E-box motif appears to be recognised by the basic helix-loop-helix leucine zipper (bHLH-ZIP) transcription factor microphthalmia (Mitf) which plays an essential role in pigment cell development, while the POU domain transcription factors Oct-1 (Bentley et al, 1994) and Brn-2/N-Oct3 (Eisen et al, 1995) bind the octamer element. Binding of Mitf and the POU-domain factors appears to be mutually exclusive: while Mitf binding at the E-box serves to initiate transcription,

binding of Brn-2/N-Oct3 at the octamer element represses tyrosinase promoter activity, possibly by displacing Mitf or by preventing its binding to the E-box (Eisen et al, 1995). The functional importance of the initiator has been demonstrated in transient transfection experiments in which mutations in the initiator region have been found to drastically reduce transcription from the human tyrosinase promoter (Bentley et al, 1994).

Another element concerned in the initiation of tyrosinase transcription is the Sp1 binding site. Sp1 is a general eukaryotic transcription factor involved in the initiation of transcription by RNA polymerase II. Bentley et al (1994) describe a non-consensus Sp1 binding site in the human tyrosinase promoter. Situated 43bp upstream of the initiator, it functions to enhance human tyrosinase gene expression. Although the motif is not conserved in other vertebrates, sequences in the same position relative to the initiator in the mouse and quail tyrosinase promoters are able to compete for Sp1 binding in gel mobility shift assays (Bentley et al, 1994). As the chicken promoter has a sequence very similar to that of the quail motif, it may likewise prove to bind Sp1 (Ferguson and Kidson, 1996). However, this has not been tested.

There remains some confusion concerning the transcription start site for the tyrosinase gene. Transcription start site analysis has revealed several (two to four) transcription initiation sites with a common start site at 80bp upstream from the ATG translation start site for the human and mouse genes, and 81bp upstream of the ATG in the quail (reviewed by Ferguson and Kidson, 1997). In the chicken, a number of potential transcription start sites have been identified by primer extension analysis; however a predominant site was not singled out, suggesting that the transcription start point for chicken tyrosinase is heterogeneous (Ferguson and Kidson, 1996).

1.6 What elements are involved in regulating pigment cell-specific expression of the tyrosinase gene?

Essentially two elements have been implicated in limiting the expression of the tyrosinase gene to melanocytes and RPE cells. The **M-box**, an 11bp motif found in the 5' sequences of all the members of the tyrosinase gene family (Lowings et al, 1992), is a positive-acting regulatory element. It contains a central E-box motif, and is positioned between 40-160bp upstream of the major transcription start site in the human and mouse genes. In the avian genes the M-box is situated slightly further upstream and is not strictly conserved over its 11bp. Further, there appear to be several other M-box like elements (termed p-MSE by Yamamoto et al, 1992) in the quail and

chick tyrosinase promoters. The functional significance of the p-MSE elements has not yet been directly tested.

A number of functional studies have established the M-box as an important component of the minimum promoter sequences required for pigment cell-specific expression of the tyrosinase and related genes, Trp-1 and DCT. As little as 270bp of the mouse tyrosinase promoter is able to drive pigment cell-specific expression of a reporter gene (Klüppel et al, 1991). However, a human promoter sequence of a similar length and which also contained the M-box, was shown to be significantly less active than the mouse fragment (Ganss et al, 1994). Further studies identified an enhancer element at about –1.86kb from the transcription start site of the human tyrosinase promoter. This element, named the **tyrosinase distal element (TDE)**, also has an E-box motif at its centre and has been shown by mutation analysis to possess protein binding ability and pigment cell specific action in the expression of the human tyrosinase gene (Shibata et al, 1992). The central bp of the human TDE are also conserved in the distal conserved region of the chicken tyrosinase promoter (Ferguson and Kidson, 1996), but the functional significance of this element in the chicken gene is not known.

There are thus three elements in the tyrosinase gene promoter that contain the E-box motif CATGTG: the initiator, the M-box and the TDE. All three elements bind the Mitf transcription factor. Thus, the question arises, what is the relative importance of the interaction of Mitf with each of these elements? In the mouse promoter, the initiator appears to be the essential element for the activation of expression by Mitf (Yasumoto et al, 1995; Bentley et al, 1994). In the human, the TDE is required in addition to the initiator, for the activation of expression by Mitf (Yasumoto et al, 1994). In both these mammalian promoters, the M-box appears only to enhance tyrosinase expression. In contrast, a 369bp quail promoter fragment that contained the Inr E-box, the Sp1 binding site and the M-box, was not able to confer tissue-specific gene expression. This implies that additional 5' flanking elements are required for cell type-specific tyrosinase activity in avians (Akiyama et al, 1994). Interestingly however, the results of a pilot study of the chicken tyrosinase promoter have indicated that an even shorter, 248bp promoter fragment (which excludes the initiator, M-box and TDE) was able to direct tyrosinase expression in RPE cells, but not in melanocytes (Ferguson, PhD thesis, unpublished results). It is not known what elements are contained in the 248bp chicken tyrosinase promoter fragment that could be responsible for mediating this difference in tyrosinase expression in melanocytes and RPE cells, or whether the results are an experimental artefact.

In addition to the M-box and the TDE, other positive and negative upstream regulatory elements have been identified in the various tyrosinase promoters; some of these appear to have a cell type-specific function and some not. An upstream activator element identified by Ganss et al (1994) in the mouse promoter overlaps a putative melanocyte-specific enhancer element, **tyrosinase element-1 (TE-1),** at –240/-216bp from the tsp, reported by Ponnazhagen and Kwon (1992). A similar sequence exists in the quail and chicken promoters, but has not been tested for enhancer function. Finally, a cell type-specific repressor region (-193/-125) in the mouse promoter indicates that the tyrosinase gene is under negative control in melanocytes (Ganss et al, 1994).

1.7 What promoter elements are necessary for wild-type levels of tyrosinase gene expression?

When transgenic animals are generated, the position of integration in the genome can affect the expression of the transgene. Thus, founder animals with different integration sites may show widely different levels of expression. A transgene is said to be position-independent when its expression level is not influenced by the site of integration but rather, is proportional to the number of copies integrated into the host genome.

Expression of tyrosinase transgenes containing up to 5.5kb of 5' flanking sequence has been shown to rescue the albino phenotype in transgenic mice (Beermann et al, 1993). However, although the transgenic mice are pigmented (indicating that the transgene carries the elements necessary for correct temporal and spatial expression), the level of pigmentation varies and wild-type levels of tyrosinase expression are not attained. Thus, the question arises, what additional elements must be required for wild-type expression levels of a transgene to be attained?

A matter of relevance to the above question is that eukaryotic DNA does not exist in a 'naked' form, but associates with histone proteins to form nucleosomes which can in turn be folded into higher-order chromatin structures. The structure of chromatin influences transcription by determining the accessibility of binding factors to the regulatory elements of a gene. Locus control regions (LCRs) are a group of elements that have been studied in this context. An LCR is a regulatory region that controls the transcriptional status of a gene locus. First described for the β-globin gene (Grosveld et al, 1987), LCRs contain elements required to establish an open chromatin structure and to insulate the gene from regulatory influences of the neighbouring chromatin. Thus, LCRs are able to confer position-independent expression of a transgene.

A dissection of the molecular lesion that gives rise to the chinchilla-mottled mouse mutation has provided useful information on the role of possible LCRs in mouse tyrosinase gene transcription. Mice homozygous for the mutation have reduced tyrosinase expression and stripes of light and dark grey fur. Porter et al (1991) found that the mutation is a rearrangement of the tyrosinase gene that separates the proximal regions of the gene from a potential regulatory region at -15kb from the transcription start point. This region consists of a DNAse-I hypersensitive site embedded within a matrix attachment region. DNAse I hypersensitive (HS) sites indicate transcriptionally active regions of chromatin, and may be useful in identifying regulatory regions of a gene of interest. Matrix attachment regions (MAR) are specific regions of DNA that associate with structural elements of the nuclear matrix. They are thought to define chromatin domain boundaries and to have functional significance in the regulation of gene activity. Thus, Porter et al (1991) speculated that separation of this potential LCR from the more proximal parts could render the gene unable to successfully achieve the open chromatin conformation necessary for wild-type levels of tyrosinase expression. This idea is supported by a recent finding by Wu et al (1997). They found that a 5.4kb intracisternal A particle (IAP) insertion in the mouse tyrosinase promoter causes somatic mosaicism (coat-colour mottling) in mice carrying the mutation. They speculate that the insertion isolates the proximal 225bp of the tyrosinase promoter from upstream LCR regulatory elements, thereby preventing those upstream elements from exerting their regulatory function on tyrosinase expression (Wu et al, 1997).

Experiments with transgenic animals have been carried out to clarify the potential LCR activity of the –15kb region identified by Porter et al (1991). Ganss et al (1994) (who calculate the LCR regulatory element to be at –12kb) found that a 200bp element coinciding with the hypersensitive site (HS) functions as a melanocyte-specific transcriptional enhancer in transgenic mice. Porter and Meyer (1994) likewise generated transgenic mice with constructs containing all, or only subfragments, of the potential LCR. They found that the HS site acts as a melanocyte-specific enhancer, while the MAR confers position-independent expression of tyrosinase. In their rescue experiments of albino mice, high levels of copy number-independent transgene expression were attained with transgenes that contained the full –15kb region. In most (but not all) mice, the transgene expression was also position-independent. They determined that both the HS site and the MAR are required for LCR activity of the transgene. Since then, Montoliu et al (1996), have used YAC transgenes to demonstrate that the HS site region contains essential elements for the correct copy number-dependent and position-independent expression of tyrosinase in transgenic mice.

1.8 Are there promoter elements that allow tyrosinase expression to be regulated differently in melanocytes and RPE cells?

The possibility that melanogenesis is regulated differently in melanocytes and RPE cells at the level of tyrosinase expression has been alluded to in section 1.3 of this review. Most interestingly, evidence from the studies mentioned above now suggests that the LCR at –15kb might play a role in regulating tyrosinase expression differently in RPE cells and melanocytes. This possibility first became known when the pigmentation levels of RPE cells and neural crest-derived melanocytes in transgenic animals were compared. Ganss et al (1994) found no difference in the levels of pigmentation of the choroid (derived from the neural crest) and the RPE in the eyes of their transgenic mice. This result implies that similar mechanisms of transgene regulation operate in tyrosinase expressing cells of different developmental origins.

In contrast, Porter and Meyer (1994) found that in the eyes of transgenic animals, the pigment cells of the choroid and anterior iris were more pigmented than the RPE. This result implies that different mechanisms may be responsible for mediating expression in RPE and neural crest-derived melanocytes. Likewise, Montoliu et al (1996) with YAC transgenes found that when the transgene lacked the HS site, most of the mice had pigmented eyes, while the coat was unpigmented or very lightly pigmented. Their results suggest that deletion of the HS region has a differential effect in eye and skin pigmentation. However, they do not specify whether the eye pigmentation they observe is the result of a pigmented RPE or of pigmentation in the neural crest-derived melanocytes of the eye (Montoliu et al, 1996). None the less, taken together these studies suggest that the –15kb LCR could play a role in regulating tyrosinase gene expression differently in RPE cells and neural crest-derived melanocytes.

A very recent report by Porter et al (1999) now confirms that the –15kb LCR is indeed the first known region in the tyrosinase 5' flanking sequences to exhibit different regulatory activities in pigment cells of different developmental origins. Using transfection experiments and mice generated with a series of transgenes containing different portions of the LCR, they identified both positive and negative elements with regulatory activities specific to subsets of pigment-producing cells. In the MAR region, they identify a negative regulatory element specific to ocular pigment cells of neural crest origin. The MAR region also appears to contain an RPE-specific enhancer region. An RPE-specific negative regulatory region is centred around the HS.

Gel shift assays using the –15kb region and nuclear extracts from different cell types yielded a further exciting (although preliminary) result. They found that different proteins interact with the enhancer core region, depending on whether neural crest derived melanoma cell nuclear extracts, or RPE nuclear extracts are used in the binding experiments (Porter et al, 1999). This is the first direct evidence of a mechanism whereby tyrosinase expression may be regulated differently in pigment cells of differing developmental origins.

1.9 How could trans-acting factors affect tyrosinase expression differently in RPE and neural crest-derived melanocytes?

A second line of evidence for different mechanisms of tyrosinase regulation in melanocytes and RPE cells comes from studies of microphthalmia, a so-called 'master regulator' of melanocyte development. Mitf has already been mentioned briefly in section 1.3. There, it was suggested that *Mitf* expression may be activated differently in melanocytes and RPE cells, because Pax3 (which was recently shown to transactivate *Mitf*, Watanabe et al, 1998) is expressed in melanocytes but not in RPE cells. A growing body of evidence now suggests that, in addition to requiring different activation signals in melanocytes and RPE cells, the role of microphthalmia may be quite different in these two populations of pigment cells.

Studies of the phenotypes of animals with mutations in microphthalmia first provided evidence that *Mitf* has different roles in RPE cells and melanocytes. Most *Mitf* alleles have effects on the RPE, however, at least one murine allele with a "black-eyed white" phenotype has been described (Kreitner, 1957). In this mouse, eye development is nearly normal but coat pigmentation is greatly reduced. Another *Mitf* mutation in mice leads to vitiligo, in which neural crest-derived melanocytes and RPE cells are affected differently. Mice homozygous for the mi^{vit} mutation show a progressive loss of hair bulb melanocytes with resultant coat colour lightening over time. In contrast to the reduction in coat melanocyte numbers, the cells of the RPE proliferate pathologically. This and other early defects later leads to severe retinal degeneration, retinal detatchment and blindness. However, while the RPE phenotype of the mutant stabilises postnatally, the coat phenotype progressively worsens (Sidman et al, 1996).

Because the mutation appears to affect melanocytes and RPE cells differently, Sidman et al (1996) have suggested that Mitf may interact with different proteins in RPE and in its other cellular targets. This idea is supported by a recent investigation by Nakayama et al (1998). They studied the

phenotypes of neural crest-derived melanocytes and the RPE in a number of different *Mitf* mutants. They too found that *Mitf* mutations lead to a decrease in neural crest-derived cell numbers, while RPE cells proliferated (Nakayama et al, 1998).

Finally, evidence from a mutation in *nacre,* a zebrafish gene coding for a bHLH-ZIP transcription factor related to microphthalmia, suggests that neural crest-derived pigment cells have a greater dependence on a functional microphthalmia protein than retinal pigment epithelial cells (Lister et al, 1999). Whether or not this is true for mammals and / or avians remains to be seen. None the less, the question arises: what mechanism could account for apparently different roles for Mitf in pigment cells of different developmental origins?

Evidence from the latest studies has now made it possible to suggest a mechanism whereby Mitf may exert its different effects in RPE cells and melanocytes. Recently it has become known that Mitf occurs in at least three different isoforms, generated by alternate splicing or differential promoter usage. The isoforms differ in the amino-terminal domains, although they share the transactivation domain and the basic helix-loop-helix leucine zipper structure (Udono et al, 2000; for review, see Yasumoto et al, 1998). Mi-A is expressed in many cell types including the RPE, where it was found to constitute 90% of *Mitf* transcripts (Amae et al, 1998). Mi-M is expressed only in melanocytes and melanoma cell lines and not in the RPE, while Mi-H is the heart-type Mitf (see Table 1.1). While both Mi-M and Mi-A have been shown to transacivate the tyrosinase and Trp-1 promoters, Mi-H does not. An 18bp insert at the 5' end of exon 6 is absent from both Mi-A and Mi-H transcripts, while Mi-M occurs as two species: some with and some without the insert. Thus, mutations in the insert would be likely to affect Mi-M (in neural crest-derived cells) but have no effect on Mi-A (in RPE cells) and Mi-H (Yasumoto et al, 1998).

Table 1.1: Expression of the three microphthalmia isoforms.

	Mi-A (minus form only)	Mi-M (plus *or* minus form)	Mi-H (minus form only)
Melanocytes	✓	✓	-
RPE	✓ (constitutes 90% of RPE transcripts)	-	-
Heart	✓	-	✓

In conclusion, there is growing evidence to suggest that Mitf behaves differently in the RPE and neural crest-derived melanocytes. It is exciting to speculate that different Mitf isoforms could provide the basis for a mechanism whereby tyrosinase expression is regulated differently in pigment cells of different developmental origins.

SECTION C: Aim of this study.

The 5' flanking region of the chicken tyrosinase gene was first cloned and sequenced by Ferguson and Kidson (1996). Alignment of the chicken sequence with those of the human, mouse, quail and turtle tyrosinase genes revealed evolutionary conserved regions containing regulatory elements previously reported to play a role in melanocyte-specific expression of the tyrosinase gene in mammals (as summarised previously, in section 1.4). In order to test the ability of the chicken sequence to drive transcription, and to assess the functional significance of various conserved elements identified in the chicken sequence, transient transfection experiments were carried out. Constructs were generated in which 2.1kb, 1.1kb, 0.5kb and 0.2kb fragments of the chicken tyrosinase 5' flanking region were linked to a luciferase reporter gene. These constructs were introduced into cultures of chicken retinal pigment epithelial cells (RPE), immortalised quail neural crest cells (MQTNC) and human liver cells (HepG2) (Ferguson and Kidson, 1996; Ferguson, PhD thesis, unpublished data).

The results of these pilot experiments may be summarised as follows. Transfections with all constructs resulted in luciferase activities significantly greater than those observed with a promoterless luciferase construct, thus confirming that the 5' flanking sequence of the chicken tyrosinase gene does possess promoter activity (Ferguson and Kidson, 1996). However, the level of expression from the various constructs differed markedly in the different cell types. In the tyrosinase-negative HepG2 cells, low levels of expression were measured with all constructs. In MQTNC neural crest cells, the highest level of reporter activity was measured with the longest (2.1kb) promoter construct (hereafter referred to as the 'full-length promoter'), with lower levels of activity obtained from the three deletion constructs. In contrast, in the pigmented RPE cells, the highest level of activity was obtained specifically with the smallest (0.2kb) promoter construct (Ferguson, unpublished data).

The results summarised above were of interest for the following reasons. Firstly, the high level of reporter activity obtained with the longest construct in the neural crest cells appeared to indicate

that the chicken tyrosinase promoter was capable of mediating pigment cell-specific expression of the gene. Secondly, since the 0.2kb promoter fragment does not contain any of the highly conserved elements thought to be responsible for tissue-specific transcription initiation of the tyrosinase gene, the role of these elements, in RPE cells at least, seemed questionable. Finally, a comparison of the results obtained in neural crest and RPE cells suggested that the chicken tyrosinase gene promoter is regulated differently in RPE and neural crest-derived pigment cells. This result may have considerable developmental significance (Ferguson, unpublished data).

However, the interpretation of the results was made difficult for the following reasons.

- Firstly, the result indicating pigment cell specificity is doubtful because of the characteristics of MQTNC cells. MQTNC was generated by immortalising quail neural crest cells with the avian *myc*-carrying MC29 retrovirus (Fauquet et al, 1990). Differentiated melanocytes were observed in some of the original immortalised cell cultures. However, the cultures were heterogeneous in nature and the cells used in pilot studies of the chicken tyrosinase promoter were not visibly pigmented. Further, Northern blot hybridisation of RNA derived from MQTNC cells was unable to detect chicken tyrosinase transcripts. Thus, it was not established whether MQTNC cells actually express tyrosinase (Ferguson, unpublished data). In addition, Myc proteins are bHLH-ZIP transcription factors and are able to transactivate genes via E-box motifs, of which there are a number in the tyrosinase promoter. Thus, it is possible that rather than reflecting pigment cell type-specific activity, the high level of reporter activity observed with the 2.1kb tyrosinase promoter construct in MQTNC cells was the result of raised levels of Myc proteins in those cells (that is, endogenous c-Myc plus v-Myc shown by immunostaining in Fig 3a and b of Fauquet et al, 1990).

- Secondly, the high level of expression obtained with the 0.2kb construct in RPE cells is not easy to interpret because of the way the 0.2kb construct was cloned. Rather than containing only a proximal promoter fragment, the 0.2kb construct in fact consists of 203bp of proximal promoter to which is linked, at its distal end, 45bp of extreme 5' promoter sequence (Ferguson, unpublished data). Thus, it is possible that the high level of promoter activity observed with the 0.2kb construct in RPE cells is due to the unfortunate placement of the 45bp next to the 203bp, rather than because of any intrinsic or 'real' property of the promoter fragment. However, whether real or not, the difference in activity obtained with the 0.2kb construct in RPE and MQTNC cells is still remarkable and suggests that different mechanisms may regulate tyrosinase expression in RPE and neural crest-derived pigment cells.

Thus, the present study was initiated in order to address the following specific questions:

1. Does the chicken tyrosinase promoter function in a pigment cell type-specific manner?
2. Is the result obtained with the shortest construct in RPE cells a real result, or is it an artefact of the promoter construct?
3. What promoter elements are responsible for mediating the difference in activity obtained with the 0.2kb construct in neural crest-derived cells and RPE cells?
4. Are there other elements in the chicken tyrosinase promoter that allow the promoter to be regulated differently in neural crest-derived cells and RPE cells?

These questions were addressed by carrying out transient transfection experiments in non-pigmented HepG2 cells, pigmented RPE cells and a pigmented neural crest-derived melanocyte line (melan-a). The cells were transfected with the chicken tyrosinase promoter constructs cloned by Ferguson (Ferguson and Kidson, 1996; Ferguson, unpublished data), as well as with new promoter constructs that were cloned specifically for the present study.

CHAPTER TWO: Materials and methods

2.1 Cell culture

Melan-a cells (spontaneously immortalised mouse melanocytes, Bennett et al, 1987) were grown in RPMI medium (Highveld Biological, SA) supplemented with 10% foetal calf serum (FCS) which had not been heat-treated (Highveld Biological, SA), and containing 100 I.U. each of penicillin and streptomycin (Highveld Biological, SA). TPA was added to the medium to a final concentration of 200nM.

A human hepatoblastoma cell line, **HepG2** (ATCC no. HB 8065) was grown in Dulbecco's modified Eagle's medium (Sigma) containing 10% FCS (not heat-treated), without antibiotics.

Primary cultures of **chicken retinal pigment epithelial (RPE) cells** were prepared according to a modification of the methods of Eguchi and Okada (1973). Briefly, fertilised chicken eggs (Black Australorp X New Hampshire Red or White Plymouth Rock X Pile Game breeds) were incubated in a humidified incubator at 37°C. After 8-10 days, embryos were removed and eyes were dissected in Ca^{2+} and Mg^{2+}-free Hanks (CMF Hanks), pH 7.4. After removal of the cornea, lens and vitreous, the eye cups were incubated for 1 hour in CMF Hanks containing 0.05% EDTA. Thereafter, eye cups were transferred back into CMF Hanks and fine forceps were used to gently dislodge sheets of RPE from the underlying sclera. Sheets of cells were collected in a small volume of CMF Hanks and gentle agitation with a Gilson pipette was used to obtain a suspension of small clusters of cells (5-10 cells per cluster). Cells were plated in Eagle's MEM (Sigma) containing 10% heat-inactivated FCS and 100 I.U. each of penicillin and streptomycin.

All cells were grown at 37°C in a humidified incubator. HepG2 and RPE cells were grown at 5% CO_2 while melan-a cells were grown at 10% CO_2. Manipulation of cells was conducted in a lamina flow tissue culture hood. Media and additives that were not purchased as sterile solutions were sterile-filtered using 0.22µm filters or autoclaved before use.

2.2 Gene constructs and cloning

The following plasmids were used as controls in transfection experiments:
pRSV-β-gal: this plasmid contains the β-galactosidase (β-gal) reporter gene under the control of a Rous sarcoma virus promoter (Edlund et al, 1985).
pGL2-control: this positive control vector, which contains the firefly luciferase coding region under the control of SV40 promoter and enhancer sequences (Promega Corporation) is referred to as **pGL2-luc** throughout this thesis.
pGL2-basic: this vector contains the firefly luciferase coding region without any promoter or enhancer sequences (Promega Corporation) and is used as a negative control for background luciferase expression due to the pGL2-basic vector backbone.
pRL-SV40 and **pRL-CMV** are reporter vectors which contain the coding region of the *Renilla* luciferase gene under the control, respectively, of the SV40 enhancer/promoter region or the CMV enhancer/promoter region (Promega Corporation).

The following chicken tyrosinase promoter-reporter constructs, cloned by Ferguson (Ferguson and Kidson, 1996; Ferguson, unpublished data) were used in transfection experiments:
Tyr2.1-Luc: this vector contains the firefly luciferase reporter coding region under the control of 2.1kb of the chicken tyrosinase promoter.
Tyr1.1-Luc, **Tyr0.5-Luc** and **Tyr0.2-Luc** contain respectively 1.1kb, 0.5kb and 0.2kb deletion fragments of the 2.1kb chicken tyrosinase promoter, linked to the firefly luciferase reporter coding region in pGL2-basic. For the remainder of this thesis, Tyr0.2-Luc is referred to as **Tyr0.248-Luc**, in order to distinguish it from a new, shorter, construct cloned in the course of the present study (Tyr0.203-Luc, see below).

For the present study, additional chicken tyrosinase promoter-reporter constructs were cloned as follows:
Tyr0.203-Luc: (see Fig 2.1) Tyr2.1-Luc was digested with *Asn*I to yield four fragments that were separated by mini agarose gel electrophoresis. The largest fragment, containing 203bp of proximal tyrosinase promoter and some 2400bp of vector sequence, was isolated from the gel by electro-elution. The "sticky" *Asn*I end of the fragment was made blunt by a filling in reaction using Klenow polymerase, in order to generate a blunt end compatible with the *Sma*I site of the vector. After phenol extraction and precipitation of the reaction products, the fragment was digested with *Hind*III to release the 203bp of promoter from the adjacent vector sequence, generating a 3' terminus compatible with the *Hind*III site of the receiving vector. The reaction products were then

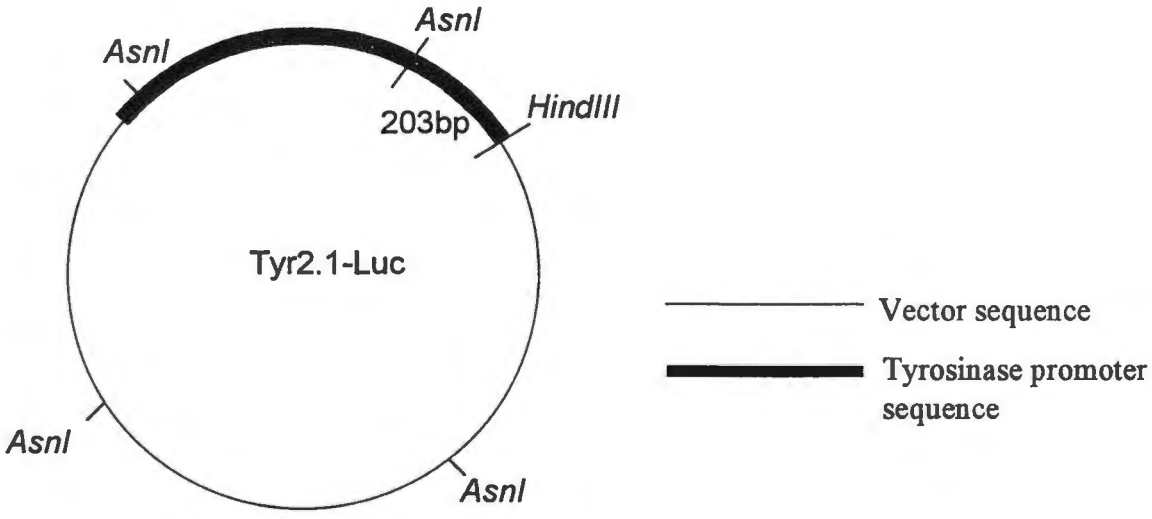
purified by phenol extraction and precipitation. The pGL2-basic vector was prepared to receive the insert by sequential digestion with *Sma*I and *Hind*III. Equimolar amounts of insert and vector were ligated overnight at 15°C. Competent *E. coli* XL1-blue cells were then transformed with the ligation reactions and plated onto freshly prepared bacterial plates. The following day, colonies were picked and minilysates prepared for restriction digest analysis of the ligation products.

Tyr0.045-Luc: (see Fig 2.2) Tyr2.1-Luc was digested with *Asn*I and *Sca*I to generate five fragments. After separation by agarose gel electrophoresis, a fragment consisting of 1230 of 5' vector sequence and 45bp of the distal chicken tyrosinase promoter was electro-eluted from the gel. The 3' overhanging *Asn*I terminus of this fragment was filled in using Klenow polymerase to generate a blunt end compatible with the *Sma*I restriction site of the vector. The reaction product was then purified by phenol extraction and precipitation. The pGL2-basic vector was prepared by sequential digestion with *Sma*I and *Sca*I, in order to remove the portion of vector sequence corresponding to that contained in the prepared insert fragment. Equimolar amounts of insert and vector were ligated at 15°C overnight. Competent *E. coli* XL1-blue cells were transformed with the ligation products. After plating onto freshly prepared bacterial plates, colonies were picked and minilysates prepared for restriction digest analysis of the ligation products.

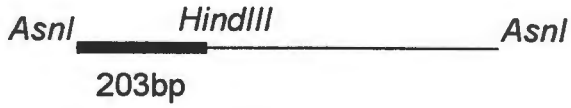
Tyr2.06-Luc: (see Fig 2.3) ExoIII 5.25 [pUC19 containing 2.1kb of chicken tyrosinase promoter, Ferguson and Kidson (1996)] was linearised by digestion with *Xba*I and purified by phenol extraction and precipitation. The 5' *Xba*I "sticky" end was filled in using Klenow polymerase, in order to generate a blunt end compatible with the vector *Sma*I site, and purified again. Digestion with *Hind*III then released the proximal 2060bp of the chicken tyrosinase promoter from the ExoIII 5.25, generating a 3' terminus compatible with the *Hind*III site of the receiving vector. This 2060bp fragment was separated out by agarose gel electrophoresis and purified from the gel using Geneclean (GENECLEAN II Kit, BIO 101 Inc.) according to the manufacturer's instructions. The pGL2-basic vector was prepared by sequential digestion with *Sma*I and *Hind*III. A 3:1 molar ratio of insert:vector was used in ligations that were performed overnight at 15°C. Competent *E. coli* XL1-blue cells were transformed with the ligation products. After plating onto freshly prepared bacterial plates, colonies were picked and minilysates prepared for restriction digest analysis of the ligation products.

Restriction digests, phenol extractions, precipitation, agarose gel electrophoresis, mini-preps of DNA, ligation reactions and transformation of bacteria were performed according to methods outlined in Davis et al, 1986.

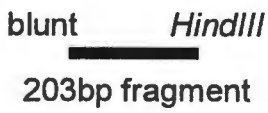
Fig 2.1: Cloning of Tyr0.203-Luc from Tyr2.1-Luc. See text for details.



1. Digest with *AsnI*, obtain four fragments
2. Elute desired fragment

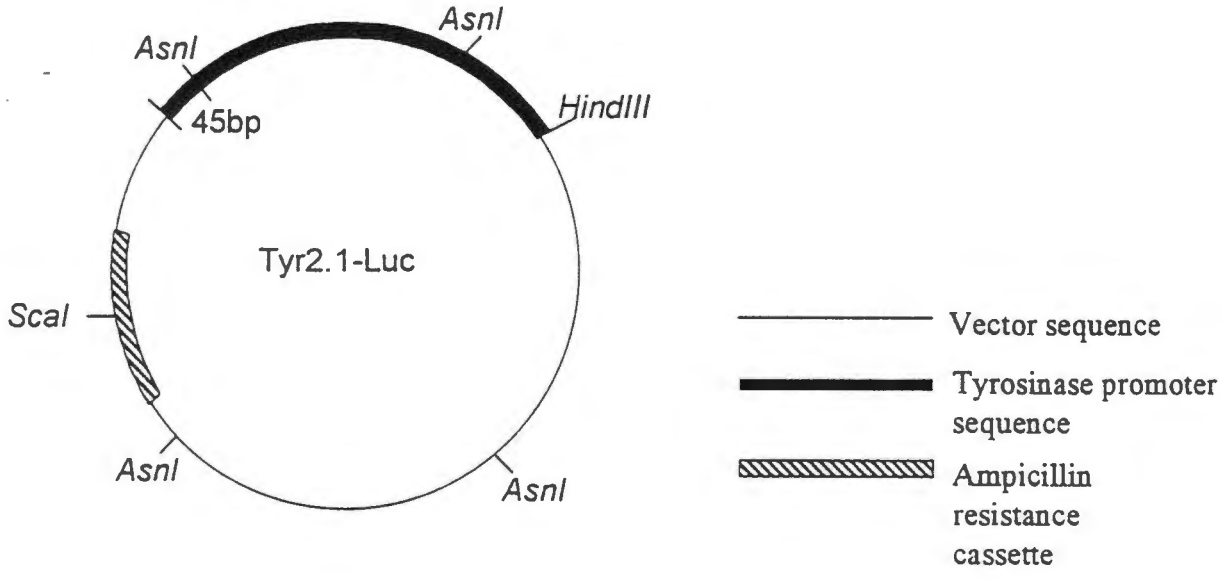


3. Blunt end with Klenow polymerase
4. Digest with *HindIII* to release 203bp fragment

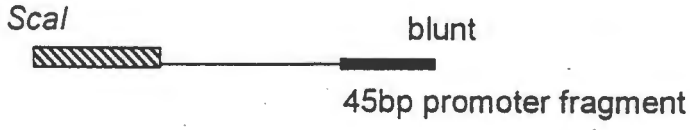


5. Ligate into *SmaI* / *HindIII*-cut pGL2-basic

Fig 2.2: Cloning of Tyr0.045-Luc from Tyr2.1-Luc. See text for details.



1. Digest with AsnI / *ScaI*, obtain 5 fragments
2. Elute desired fragment
3. Blunt end with Klenow polymerase



4. Ligate into *ScaI* / *SmaI*-cut pGL2-basic to restore Ampicillin resistance cassette

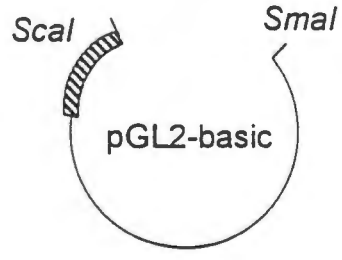
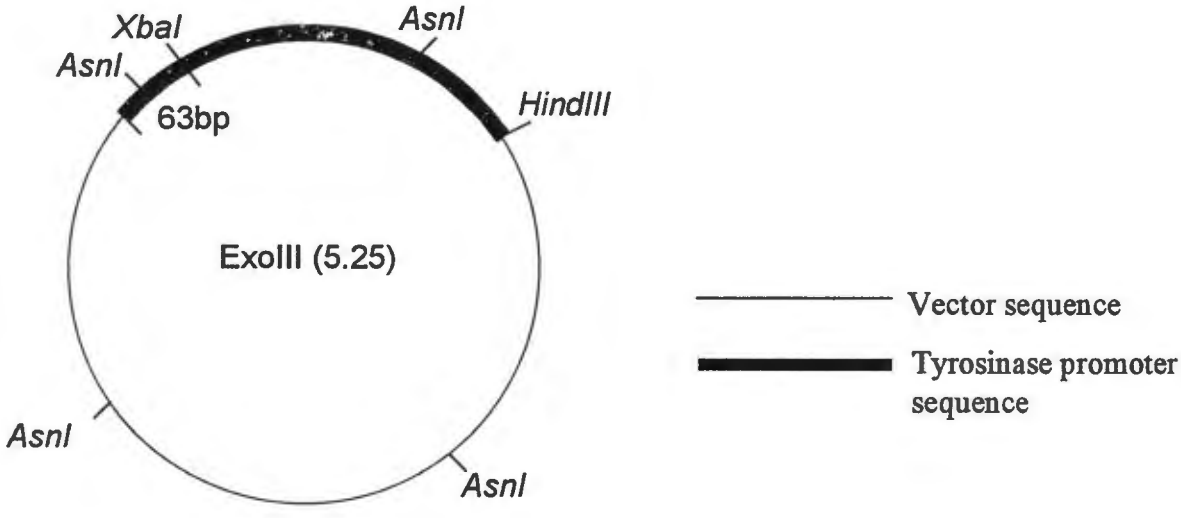
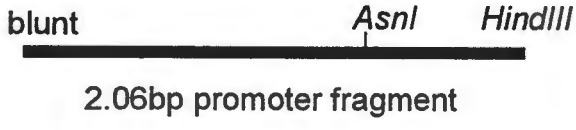


Fig 2.3: Cloning of Tyr2.06-Luc from ExoIII (5.25). See text for details.



1. Digest with *XbaI* to linearise vector
2. Blunt end with Klenow polymerase
3. Digest with *HindIII*
4. Elute desired fragment



5. Ligate into *SmaI* / *HindIII*-cut pGL2-basic

2.3 Caesium chloride-ethidium bromide purification of plasmids

Prior to transfection plasmid DNA was prepared by the alkaline lysis maxi-preparation procedure and purified by ultracentrifugation on a caesium chloride-ethidium bromide gradient, according to the methods in Sambrook et al (1989), with minor modifications. Briefly, 5ml of Luria Broth (LB) containing ampicillin was seeded from frozen stocks of *E. coli* carrying the appropriate plasmid and incubated overnight in a waterbath at 37°C with continual agitation. The following day, 1ml of the turbid starter culture was added to 500ml of LB with ampicillin and again cultured overnight at 37°C in an orbital shaker. Chloramphenicol was added to the culture to a final concentration of 170 µg/ml when OD_{600} of the culture was approximately 0.4 (at between 6 and 10 hours). The following day the turbid culture was centrifuged at 5000 x g for 10' at 4°C, to pellet the bacteria. Pellets were resuspended in a total of 6ml glucose buffer and lysed by the sequential addition of 12ml of freshly prepared NaOH/SDS and 9ml potassium acetate solution (Appendix 1). A pellet of bacterial debris was formed by centrifugation at 12 000 x g for 10' at 4°C. The supernatant was collected and the plasmid DNA precipitated by the addition of an equal volume of isopropanol and standing at –80°C for 10'. DNA pellets were collected by centrifugation at 12 000 x g for 10' at 4°C, air dried for one hour and resuspended in a total of 6ml dH_2O.

The CsCl/EtBr gradient was set up as follows: approximately 6g of CsCl were added to the DNA solution and allowed to dissolve completely. The density of the solution was adjusted to between 1.58 and 1.63g/ml, by the addition of CsCl or dH_2O as appropriate. Then, 480µl of EtBr (10mg/ml stock solution) was added and the solution was centrifuged at 12 000 x g for 10' at 4°C to allow a scum or pellet of bacterial protein to form. The clear DNA/CsCl/EtBr solution was then carefully collected using a large bore needle and loaded into Quickseal tubes (Beckman) which were heat sealed after the tubes were balanced to within 0.01g. The tubes were loaded into a Vti65 rotor and centrifuged at 55 000rpm with slow acceleration and no brake, at 25°C for 16 to 20 hours using a Beckman L-70 ultracentrifuge.

The following day, bands of DNA in the tubes were visualised with UV light. DNA was collected in as small a volume as possible from the lower (closed circular plasmid) band using a large bore needle. EtBr was removed from the DNA by repeated extraction with an equal volume of H_2O-saturated n-butanol and the final aqueous solution was precipitated for 30' at room temperature by the addition of 2 volumes dH_2O and 3 volumes of isopropanol. DNA pellets were collected by centrifugation at 12 000 x g for 10' at 4°C, washed with 70% EtOH, air dried and resuspended in an appropriate volume of dH_2O (500µl to 2ml). A spectrophotometric quantitation was carried out and

the identity of the plasmid confirmed by restriction enzyme digest and agarose gel electrophoresis prior to use of the plasmid in transfection experiments.

2.4 Transient transfections

Calcium phosphate precipitation transfections were carried out according to the methods of Gorman et al (1982) and Kingston (1987). HepG2 cells were seeded into six-well tissue culture plates at 1.02 x 10^5 cells per 35mm well, 24 hours prior to transfection. Culture medium was replaced with 2ml fresh medium 4 to 8 hours before transfection. Fine, milky calcium phosphate-DNA precipitates were prepared by mixing 'Solution A', containing DNA and $CaCl_2$ with 'Solution B' containing 2 x HBS (pH 7.1) and 100 x PO_4 under a continuous stream of air bubbles (Appendix 1). The precipitate was allowed to stand at room temperature for 30 minutes, after which it was mixed with vigorous pipetting and added dropwise (200 µl/dish) over the surface of the medium covering the cells. The cells were exposed to the precipitate overnight for approximately 18 hours after which the medium containing the precipitate was removed, the cells were rinsed twice using phosphate-buffered saline (PBS) and fed with 2ml fresh medium. At 48 hours post-transfection, cells were washed twice with PBS and lysed *in situ* by the addition of 200-500µl of 1 x Passive Lysis Buffer (Promega Corporation). Cell debris was scraped to one side of the dish using a rubber policeman, and transferred to an eppendorf tube. Lysates were subjected to 2 freeze-thaw cycles to accomplish complete lysis of the cells, and the cell debris was then pelleted by centrifugation in a microfuge at 12 000 x g for 1 minute at room temperature. The supernatant fluid was transferred to a fresh tube after which it was either assayed directly for reporter gene activity, or frozen at –20°C for no longer than one month.

RPE and melan-a cells were transfected using the lipidic transfection reagent, **FuGENE 6 Transfection Reagent** (Boehringer Mannheim), according to the manufacturer's instructions. RPE cells were plated directly from dissection into 6 well tissue culture plates, using (approximately) the cells obtained from one eye per 35mm well. Cells were fed with fresh medium the day after plating and again on or before the day they were transfected, 4 to 7 days after plating. Melan-a cells (passage numbers 25 to 28) were plated at a density of 2 x 10^5 cells per 35mm well, 24 hours prior to transfection. A DNA: FuGENE 6 complex was prepared by adding FuGENE6 reagent (diluted with serum-free cell culture medium) to plasmid DNA at a ratio of 3µl undiluted FuGENE 6 reagent to 2µg plasmid DNA. The mixture was allowed to stand at room temperature for 15 minutes, before adding the DNA-FuGENE complex dropwise over the surface of the cells. Cells

were fed with fresh medium the day after the addition of the DNA-FuGENE 6 complex and harvested 48 hours post transfection, as described for HepG2 cells (above).

In all experiments, transfections were performed in duplicate for each luciferase plasmid to be tested. After various experiments were carried out to establish optimal transfection conditions, cells were co-transfected with 0.5µg firefly luciferase plasmid (chicken tyrosinase promoter constructs or pGL2-luc or pGL2-basic) and 0.05µg pRL-CMV luciferase plasmid. The following controls were included: To control for efficiency of uptake of DNA in different dishes in a single experiment, the luciferase plasmids were co-transfected with either pRSV-*β*gal (in initial pilot experiments carried out to establish transfection procedures) or (in later experiments) with pRL-SV40 or pRL-CMV. To control for transfection efficiency between different cell types, one set of duplicate dishes in each transfection experiment was transfected with the strongly expressed pGL2-Luc. To control for background luciferase expression, one set of duplicate dishes was transfected with the pGL2-basic vector. To control for possible background expression of the co-transfected *Renilla* luciferase plasmid (pRL-SV40 or pRL-CMV), one set of duplicate dishes was left untreated in each experiment.

2.5 Reporter gene assays

Protein concentrations were determined using the Bio-Rad Protein Assay, according to the manufacturer's instructions. BSA made up in 1 x Passive Lysis Buffer was used as a protein standard. In initial transfection experiments where cells were transfected with pRSV-*β*gal, *β*-galactosidase activity in cell lysates was assayed according to Herbomel et al (1984). Likewise, in initial experiments where cells were transfected with only a firefly luciferase reporter (rather than with both a firefly and a *Renilla* luciferase vector), luciferase activity was measured using either the Luciferase Reporter Gene Assay kit (Boehringer Mannheim) or a Promega Luciferase Assay Kit (Promega Corporation), according to the manufacturer's instructions.

In subsequent experiments, cells were transfected with both a firefly reporter (driven by the promoter of interest) and a *Renilla* reporter construct (to act as an internal control for transfection efficiency). In these experiments, the activities of firefly and *Renilla* luciferase were measured sequentially from a single sample of the lysate (Fig 2.4) using Promega's Dual-Luciferase Reporter Assay System (Promega Corporation) and a luminometer (Bio-Orbit 1253 luminometry system). First, firefly luciferase activity was measured by combining 20µl of cell lysate with 100µl of

Luciferase Assay Reagent II (LARII), which contains the firefly luciferase substrate, luciferin. After measuring the luminescence (light units) by means of a luminometer, a second reagent was added to the same sample tube. This reagent, Stop & Glo, both quenches the firefly luciferase reaction and contains coelenterazine, the substrate for *Renilla* luciferase. Light emission generated by this reaction was measured to determine the activity of the *Renilla* control plasmid.

2.6 Calculation of Relative Luciferase Activity

In order to compare information obtained from different experiments and using different cell types, each result was finally expressed as a relative luciferase activity (RLA). RLA was calculated as follows. First, 'light units / mg protein' was calculated by dividing the values (light units) obtained for both firefly and *Renilla* luciferase activity for each cell lysate by the protein concentration (mg/ml) measured for that lysate. Then, firefly luciferase activity (light units / mg protein) was normalised by dividing by the internal control *Renilla* luciferase activity (light units / mg protein). Finally, the normalised activity measured from each experimental plasmid was expressed as a percentage of the activity of the constitutively expressed positive control vector, pGL2-Luc, where the activity obtained with pGL2-Luc was assigned a value of 100%.

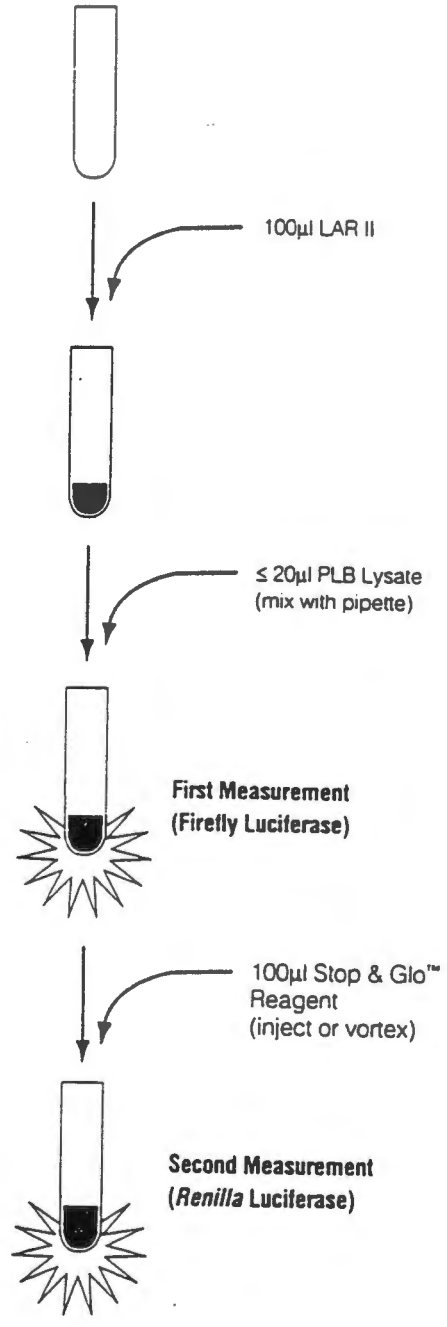


Fig 2.4: Sequential measurement of firefly and *Renilla* luciferase activities from a single sample of cell lysate. Luciferase Assay Reagent II (LAR II), containing the substrate for firefly luciferase, is added to the lysate and a first measurement taken. Then Stop & Glo reagent is added to both quench light emission by the firefly luciferase reaction and initiate the reaction of *Renilla* luciferase. After Promega Corporation, 1998.

CHAPTER THREE: Results

The aim of the present study was to examine the regulation of expression of the chicken tyrosinase gene in pigment cells of different developmental origins. This was achieved using a series of deletion constructs of the chicken tyrosinase promoter. The promoter fragments were linked to a luciferase reporter gene and transient transfections were carried out in which the constructs were introduced into RPE cells and melanocytes. The activity of the tyrosinase promoter was analysed by assaying luciferase reporter gene activity in lysates of the transfected cells.

Before the above transfection experiments could be carried out a number of technical obstacles had to be overcome. These were:

- the selection and successful manipulation of appropriate cell lines
- the establishing of effective transfection procedures for each of the chosen cell lines and,
- the selection of a suitable second reporter gene to act as an internal control for transfection efficiency in the experiments to follow.

3.1 Choice of cell lines and cell culture.

The planned transfection experiments required that pigmented cell lines of both neuroepithelial and neural crest origins be cultured. In addition, a line of non-pigmented cells was needed to serve as a negative control cell line.

Primary cultures of chick RPE cells

Chicken RPE cells are routinely cultured in this laboratory according to a modification of the method described by Eguchi and Okada (1973). However, when first attempting to culture and subculture RPE cells for use in transfection experiments, a number of difficulties arose. Briefly, these problems were as follows:

1. Over time, the cells start to dedifferentiate and become less pigmented. This well documented phenomenon (Itoh and Eguchi, 1986) is obviously not desirable for experiments that aim to study the expression of genes responsible for maintaining the differentiated state of RPE cells (such as tyrosinase).

2. Poor plating efficiency: subcultured cells had a very poor plating efficiency and were slow to proliferate. Plating for transfection experiments usually requires that cells are counted, and a specific number of cells plated into the experimental wells for transfection with different vector constructs. In order to facilitate accurate counting of the cells using a haemocytometer, it is necessary to thoroughly disaggregate clumps or sheets of cells using a trypsin / EDTA solution. Unfortunately, RPE cells that have been treated in this way were found to have an even poorer plating efficiency than usual. An attempt was made to circumvent this problem by plating a higher number of cells than ultimately desired. However, the plating efficiency was found unpredictable and seemed to vary from culture to culture, so that sometimes the cells plated very poorly and sometimes fairly well. Furthermore, once plated, the cells did not all proliferate at the same rate. Some dishes became confluent within days while in others, the cells proliferated very slowly.

3. Slow growth rate: after an initial 'spurt', plated RPE cells have a relatively slow growth rate. This phenomenon seemed to be exacerbated when the cells have been subject to manipulations of the kinds mentioned above. Altogether, it could take weeks of culturing the cells to prepare for a single transfection experiment.

All these factors combined to make it extremely difficult to prepare multiple wells of cells with a desired degree of confluence on the same day. Thus, additional experiments were carried out in which refinements to the RPE cell culture method were designed and tested. The following modifications were developed:

1. In order to circumvent the problem of dedifferentiation, cells were used for transfection within as few days as possible from the day of the dissection. On average, transfections were performed on cells that had been 6 days in culture. These cells were proliferating but still retained their typical "cobblestone" morphology and were highly pigmented.

2. In order to overcome the problems of poor plating efficiency and slow growth rate, passaging was avoided. Rather, freshly dissected RPE cells were plated directly into the six-well plates in which the transfection experiments were carried out. Rather than using enzyme to disaggregate the sheets of RPE cells, a Gilson pipette was used to disperse the cells mechanically by gently pipetting up and down using a 1000µl or a 200µl plastic tip. Cells were found to plate somewhat better after this treatment than after treatment with enzyme.

3. In order to avoid passaging as far as possible, it was also necessary to solve the problem of counting the cells. Thus, to obtain similar numbers of cells in each well, roughly one eye was dissected per well plated. Therefore, if sixteen wells were required for the experiment, 12 to 20 eyes were dissected (0.75 to 1.25 eyes per well). In this way, extreme dispersion of the cells in order to count them was avoided.

The cultures prepared in this way had a good plating efficiency and proliferated well. Using the "one eye per 35mm well" rule of thumb, pigmented cultures were obtained which were approximately 50% confluent after four days to one week in culture, when they were used for transfection experiments. The cells were still highly pigmented and retained their characteristic cobblestone morphology on the day of harvesting.

Cell culture of neural crest-derived melanocytes

Ideally, the analysis of the chicken tyrosinase promoter would be performed using a chicken neural crest-derived melanocyte line. However, such a cell line does not exist. Thus, a series of neural crest culture experiments was carried out according to the methods of Boissy and Halaban (1985), with minor modifications, in an attempt to prepare primary cultures of chick melanocytes. The success of this method depends on the use of a selective medium to enhance the survival of melanocytes and bring about the simultaneous death of contaminating, non-pigmenting cells. Unfortunately, several attempts to generate pure, proliferative cultures of neural crest-derived melanocytes were unsuccessful. Essentially, in the 'selective medium', which is supposed to enhance melanocyte survival, both non-pigmented contaminating cells and melanocytes lifted from the substrate. This resulted in a constant loss of all cell types and soon few or no viable cells were left (results not shown).

Epidermal derived melanocytes

Melan-a is a spontaneously immortalised line of pigmented melanocytes derived from normal epidermal melanoblasts of C57BL mice (Bennett et al, 1987). Many groups have used this non-tumorigenic and well-characterised cell line for studying the normal expression of melanogenic genes from a variety of species. Therefore, melan-a was selected as an alternative to chick melanocytes derived directly from the neural crest for transfection experiments.

Negative control cell line

As a non-pigmented negative control cell line, the human hepatoblastoma line, HepG2, was selected. Previously, it has been shown by Northern blot hybridisation analysis that HepG2 cells do

not express tyrosinase (Kwon et al, 1987). Apart from its lack of pigmentation, HepG2 was chosen for the ease with which it can be cultured, and because it is readily transfected by means of the relatively inexpensive $CaPO_4$-DNA co-precipitation method.

3.2 Establishing transfection procedures.

The experimental introduction of DNA into cells may be accomplished by a variety of different methods. These transfection methods vary with respect to transfection efficiency, toxicity, expense and convenience. In addition, the ability of a method to mediate DNA transfer frequently varies for different cell types. Thus, a first object in transfection experiments is to identify a method (or more than one method) that is able to mediate DNA transfer in the cell lines of interest. Once it has been established that the chosen method works, it is usually necessary to carry out further experiments in which the optimal transfection conditions for each line are determined.

The calcium phosphate ($CaPO_4$)-DNA co-precipitation method is the most commonly used transfection method. This method has the advantage of being relatively inexpensive and is able to mediate transfection in many cell types. Thus, a series of pilot transfection experiments were carried out to determine whether the $CaPO_4$ method could be used to transfect the cell lines chosen. To carry out the pilot experiments two different reporter constructs were used: pRSV-β-gal and pGL2-luc, which drive strong, constitutive expression of a LacZ and a firefly luciferase reporter, respectively.

Transfection of HepG2 and RPE cells with the $CaPO_4$-DNA co-precipitation method

The standard $CaPO_4$ transfection method (see Materials and Methods) was used to successfully transfect HepG2 cells (results not shown). It was also found that RPE cells were efficiently transfected by means of the $CaPO_4$ method. However, the heavy precipitate that formed adversely affected the morphology and growth of RPE cells, particularly when cells were exposed to the precipitate overnight. In extreme cases, the cells were even seen to lift from the substrate. Therefore, the length of time that the cells were exposed to the precipitate was optimised. It was found that transfection was mediated even when the precipitate was left on the cells for only four to six hours (results not shown).

Transfection of melan-a cells with the $CaPO_4$-DNA co-precipitation method

It was found that melan-a cells could not be transfected by means of the $CaPO_4$ method. Numerous transfection experiments were performed in an attempt to transfect melan-a cells by means of this method. Several transfection parameters, including seeding density of the cells, amount of plasmid transfected and length of time the cells were exposed to the precipitate, were tested without success. In addition, a glycerol shock step was added after a glycerol sensitivity test had been carried out on the cells. Because melan-a cells are routinely grown at a lower pH than other cells types used, the pH of the cell culture medium was monitored to ensure that it lay within the optimal rage for formation of the $CaPO_4$ precipitate. A modified $CaPO_4$ method (Chen and Okayama, 1987) was also attempted.

Although the transfections were unsuccessful, the experiments (in which the *β*-galactosidase reporter reporter plasmid, pRSV-*β*-gal, was used) did yield useful information regarding endogenous *β*-galactosidase activity in melan-a cells. The results of a representative experiment are summarised in the table below.

Table 3.1: Summary of results obtained in a representative $CaPO_4$ transfection experiment of melan-a cells.

Treatment of cells			*β*-galactosidase activity measured (OD_{420}) *	Morphology of melan-a cells
	$CaPO_4$ precipitate added	PRSV-*β*gal plasmid added		
Experimental cells	✓	✓	0.027	Morphology disturbed: cells very spindly and refractile
'Mock transfected' cells	✓	✗	0.048	Morphology disturbed as above
Untreated control cells	✗	✗	0.237	Normal morphology, cells more confluent than in dishes with $CaPO_4$ precipitate

* In all cases, *β*-galactosidase activity was detectable only after the assay reaction had been allowed to proceed overnight, a procedure that is not recommended for this assay as it may result in high levels of background *β*-galactosidase activity.

The following conclusions were drawn from the results summarised above. Firstly, the high level of background in both the mock transfected and untreated negative control cells suggested the presence of endogenous β-galactosidase activity in melan-a cells. Secondly, a comparison of mock transfected and untreated control cells showed altered morphology and decreased proliferation in the mock transfected cells. This suggests that the $CaPO_4$ precipitate is toxic to melan-a cells. The apparent toxicity may explain why background β-galactosidase activity was higher in the untreated cells than in the mock transfected cells.

In summary, all attempts to efficiently transfect melan-a cells by means of $CaPO_4$-DNA co-precipitation were unsuccessful. In addition, it appeared that the $CaPO_4$ precipitate was toxic to melan-a cells. Finally, it was revealed that melan-a cells express low levels of endogenous β-galactosidase activity. Certain mammalian cells have endogenous lysosomal β-galactosidase activity (Promega Corporation, 1996), and it is possible that cells with melanosomes, which are derived from lysosomes, may likewise have endogenous β-galactosidase activity.

Melan-a cells are successfully transfected with FuGENE 6 transfection reagent

Lipidic transfection reagents represent a new class of reagents that have proved to be an important addition to the repertoire of available DNA-transfection methods. FuGENE 6 is a lipidic, non-liposomal transfection reagent that was shown to mediate high levels of transfection with minimal cytotoxicity for many cell lines (Boehringer-Mannheim). A series of experiments was therefore initiated to determine the suitability of FuGENE 6 as a transfection reagent for melan-a cells.

Because these experiments were performed concurrently with $CaPO_4$ experiments, endogenous expression of β-galactosidase in melan-a cells was not yet confirmed. Thus, the plasmid pRSV-βgal was used in the initial experiments. As before, the β-galactosidase assay was allowed to proceed overnight. The results obtained were similar to those obtained with the $CaPO_4$ transfections, in that high levels of β-galactosidase activity were measured in both the mock transfected (FuGENE 6 only) and untreated control cells (results in Appendix III, Table 1). This again suggested the presence of endogenous β-galactosidase activity in melan-a cells. In contrast to the results obtained with $CaPO_4$ however, a higher level of β-galactosidase activity was measured in the experimental cells than in the control cells. This encouraging result indicated that DNA uptake had been mediated by FuGENE 6 transfection reagent. In addition, the morphology, proliferation and background β-galactosidase activity in the mock transfected and untreated control cells was very similar. This indicated that FuGENE 6 transfection reagent was not toxic to melan-a cells.

The ability of FuGENE 6 transfection reagent to mediate transfection of melan-a cells was subsequently confirmed in experiments which used the constitutively expressed luciferase reporter plasmid, pGL2-luc. In addition, a construct consisting of the chicken tyrosinase promoter and the luciferase reporter (Tyr2.1-Luc) was found to drive measurable levels of luciferase reporter activity. Finally, an experiment was carried out as recommended by the manufacturers in order to determine the optimum ratio of DNA (μg):FuGENE 6 (μl) for future transfections. Optimum reporter activity was measured when a ratio of 2:3 [DNA (μg) : FuGENE 6 (μl)] was used (see Appendix III, Table 2).

Transfection of RPE cells with FuGENE 6 transfection reagent

As mentioned previously, the $CaPO_4$ method was able to mediate transfection in RPE cells. However, the $CaPO_4$ precipitate appeared to have a deleterious effect on the morphology and growth of these cells. In the light of the successful transfection of melan-a cells with FuGENE 6 transfection reagent, an experiment was performed in order to determine whether RPE cells could be transfected with FuGENE 6 reagent. The results of this experiment indicated that, as for melan-a cells, FuGENE 6 reagent effectively mediated DNA-transfer into RPE cells without any adverse effects on the cells being observed (results not shown). Therefore, in all subsequent experiments RPE cells were transfected with FuGENE 6 transfection reagent.

The Dual-Luciferase reporter assay system

Transfection efficiency varies from experiment to experiment and even between the dishes of cells within a single experiment. This is true even when all the experimental conditions, such as number of cells, amount of plasmid and transfection method, are apparently constant. Therefore, it is necessary to include an 'internal control' to measure the efficiency of DNA uptake in different dishes in a single experiment. This is achieved by co-transfecting each dish with two plasmids: the plasmid of interest as well as a second constitutively expressed reporter plasmid. The plasmids are transfected at ratios of experimental plasmid (μg) : control plasmid (μg) of 10:1 to 50:1 (or greater). The activity of the experimental plasmid reporter is then normalised to the activity of the internal control plasmid. This serves to control for experimental variability in transfection efficiency.

pRSV-βgal is widely used as an internal control for transfection efficiency. However, the results of pilot transfection experiments in melan-a cells (described above) indicated that these cells express endogenous β-galactosidase. Therefore, an alternate reporter gene was sought to act as a control for transfection efficiency. The firefly (*Photinus pyralis*) luciferase enzyme has previously been used as an ideal reporter protein for a promoter of interest, as there is no endogenous luciferase activity

in mammalian and avian cells. Now, the gene for sea pansy (*Renilla reniformis*) luciferase has been cloned and a vector developed for the use of this enzyme as an internal control reporter gene (Promega). Both the firefly and *Renilla* luciferase enzymes catalyse reactions that result in the emission of light. However, the enzymes have dissimilar structures and substrate requirements. These differences make it possible to discriminate between their respective bioluminescent reactions. Promega Corporation has now used these enzymes to develop a highly sensitive, 'one-tube' dual-luciferase reporter assay system (see Materials and Methods for details). Essentially, the assay procedure involves adding first the firefly luciferase substrate (luciferin) and then the substrate for *Renilla* luciferase (coelenterazine) to a sample of cell lysate. Light emission is measured after each substrate is added, in order to determine the activity of the promoter of interest and the internal control. The many advantages of this system include sensitivity, absence of background and convenience. In particular, the extreme sensitivity of the system makes it most suitable for quantifying the activity of relatively poor promoters. In addition, experiment : control plasmid ratios of 50:1 or greater can be used. This helps to ensure independent gene expression of the experimental and control reporter genes.

Trial transfection experiments carried out in HepG2 cells to test the dual-luciferase assay system were successful (results not shown). In initial experiments, pGL2-luc was co-transfected with the *Renilla* vector, pRL-SV40, at a ratio of 50:1. In all later experiments, experimental plasmids were co-transfected with the *Renilla* vector, pRL-CMV, at a ratio of 100:1.

3.3 Expression of the full-length tyrosinase promoter in melan-a, RPE and HepG2 cells.

Having established transfection procedures in suitable pigmented and non-pigmented cell lines it was now possible to address the main aims of the present study. It has previously been shown that the 5' regulatory regions of the chicken tyrosinase gene function as a promoter in avian neural crest derived cells (Ferguson and Kidson, 1996). However, because of the limitations of the cell lines used in that study, it was questionable whether pigment-cell specificity of the promoter had been adequately demonstrated (refer to Chapter 1, section C, for further details). Thus, the first aim of the present study was to demonstrate conclusively that the chicken tyrosinase promoter functions in a tissue specific manner. That is, does the promoter preferentially drive reporter gene activity in pigmented cells, rather than in non-pigmented cells? This was addressed in the following manner.

Duplicate dishes of melan-a, RPE or HepG2 cells were transfected with Tyr2.1-Luc, a construct containing the 2.1kb of the chicken tyrosinase promoter region linked to the firefly luciferase reporter gene (Ferguson and Kidson, 1996). To control for transfection efficiency, Tyr2.1-Luc was co-transfected with a constitutively expressed *Renilla* luciferase reporter gene, either pRL-SV40 or pRL-CMV, at a ratio of 50:1 or 100:1, respectively. Positive and negative controls (as described in Materials and Methods) were included in each experiment. Forty-eight hours after transfection, the cells were harvested and the lysates were assayed for reporter gene activity. The experiment was repeated 11 times for melan-a cells, 9 times for RPE cells and 8 times for HepG2 cells. The results of individual experiments are represented graphically in Appendix III, Figures 1-3, and the combined results are represented graphically in Figure 3.1. The following results were obtained:

1. A low level of reporter activity was detected in non-pigmented HepG2 cells.
2. The level of reporter activity observed in pigmented melan-a cells was, on average, 7 times that observed in HepG2 cells.
3. The expression of the reporter gene in the pigmented RPE cells was, on average, 8 times that measured in HepG2 cells. However, the reporter activity observed in RPE cells showed considerable variation from experiment to experiment.
4. Background reporter expression generated from the promoterless control vector, pGL2-basic, was negligible.

These results convincingly demonstrate that the chicken tyrosinase promoter is significantly more active in pigmented cells than in non-pigment-producing cells. It was concluded that the promoter functions as a tissue specific promoter.

3.4 Expression of tyrosinase promoter deletion constructs in melan-a, RPE and HepG2 cells

To begin to analyse whether there are specific elements in the chicken tyrosinase promoter that might play a role in regulating tyrosinase gene expression, a series of deletion constructs was cloned and used in a pilot study of promoter function (Ferguson and Kidson, 1996; Ferguson, unpublished data). The deletion constructs are represented diagrammatically in Fig 3.2. **Tyr2.1-Luc** contains the entire 5' flanking sequence cloned to date for the chicken tyrosinase gene. **Tyr1.1-Luc** excludes the TDE-like element. **Tyr0.5-Luc**, contains the proximal 504nt of 5' flanking sequence. **Tyr0.248-Luc** (previously named Tyr0.2-Luc by Ferguson) excludes the putative M-box, Sp1 binding site and Inr element. It should be noted that Tyr0.248-Luc contains, at

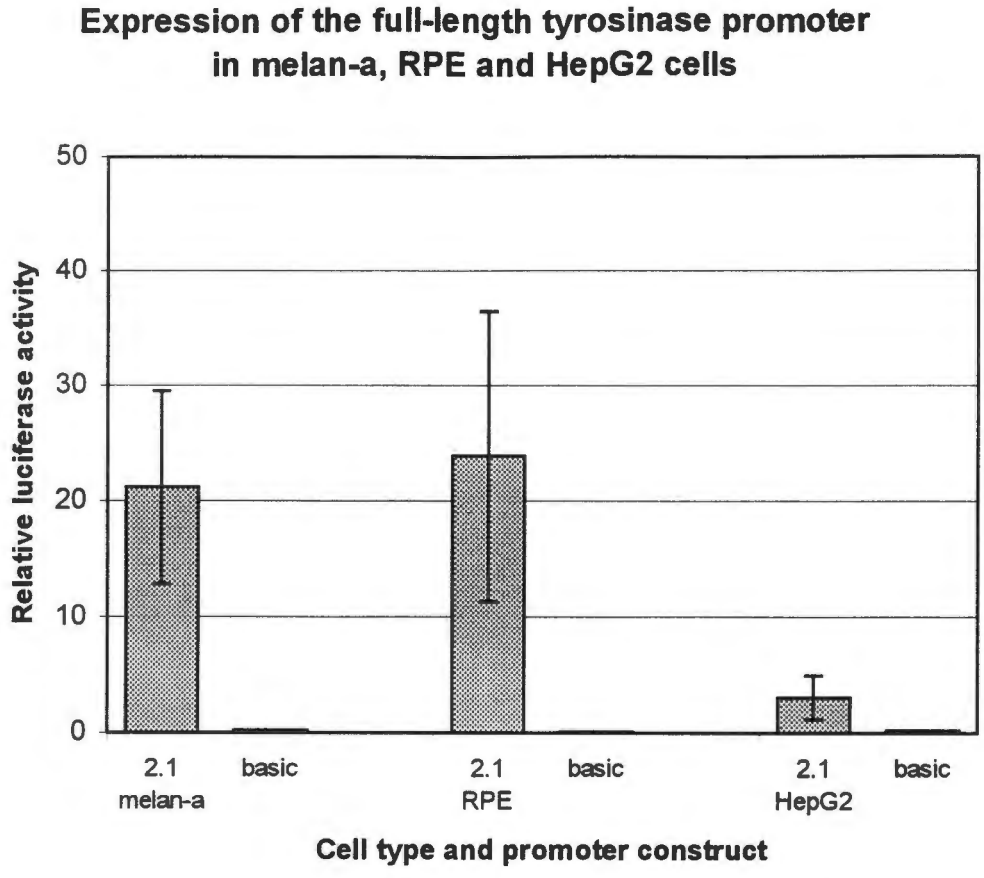


Fig 3.1: Activity of the full length chicken tyrosinase promoter construct, Tyr2.1-Luc, in melan-a, RPE and HepG2 cells. The results of 11, 9 and 8 experiments are combined for melan-a, RPE and HepG2 cells respectively. Results were normalised with respect to *Renilla* luciferase activity in the same extract and are expressed as a percentage of the luciferase activity obtained with the positive control vector, pGL2-Luc. The figures 2.1 and basic on the x-axis represent the constructs Tyr2.1-Luc and pGL2-basic, respectively.

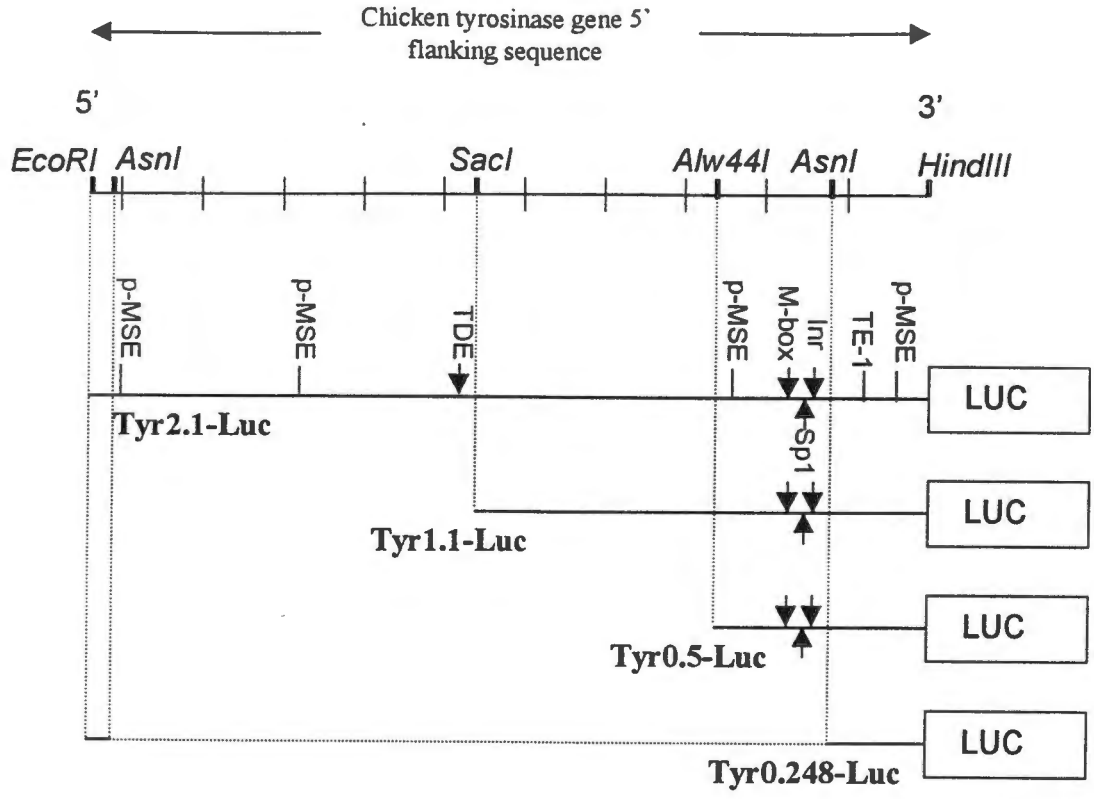


Fig 3.2: Maps of tyrosinase promoter-luciferase reporter deletion constructs used for transient transfection. After Ferguson, unpublished data.

Tyr2.1-Luc contains the entire 5' flanking sequence cloned to date for the chicken tyrosinase gene. **Tyr1.1-Luc** excludes the TDE-like element. **Tyr0.5-Luc**, contains the proximal 504nt of 5' flanking sequence. **Tyr0.248-Luc** excludes the putative M-box, Sp1 binding site and Inr element. It should be noted that Tyr0.248-Luc contains, at its distal end, 45bp from the 5' end of the promoter. This is because of the cloning strategy used to generate this construct (Ferguson, unpublished data).

its distal end, 45bp from the 5' end of the promoter. This is because of the cloning strategy used to generate this construct (Ferguson, unpublished data). The results obtained in these pilot studies were not easy to interpret, for reasons that are outlined in Chapter 1, section C. Briefly, the cell line (MQTNC) that was used to represent neural crest-derived melanocytes was unsuitable because it is in fact undifferentiated, and is transformed with myc, which can transactivate genes via E-box motifs (of which the tyrosinase promoter contains a number). In addition, a most unexpected result was obtained with Tyr0.248-Luc in RPE cells, where a high level of reporter activity was obtained with this shortest promoter construct. This result could have important consequences for our understanding of tyrosinase gene regulation. For this reason, it was important to repeat the experiments, using improved cell lines and transfection procedures.

Transfection experiments were carried out in which the tyrosinase deletion constructs were transfected into melan-a, RPE and HepG2 cells. As before, a *Renilla* luciferase reporter was co-transfected to act as an internal control. Positive and negative controls were included in each experiment. The experiments were repeated 4, 5 and 3 times for melan-a, RPE and HepG2, respectively. The results of experiments for the different cell types are presented in Figures 3.3 to 3.5. Examination of the results revealed interesting differences in the patterns of reporter activity in melan-a, RPE and HepG2 cells.

In **melan-a** cells the following was observed:

1. The highest level of activity was obtained with the full-length promoter construct.
2. The activity obtained with Tyr1.1-Luc was approximately half that obtained with the full-length construct. The most likely explanation for this result is that there is a positive regulatory element in the promoter sequences upstream of the 1.1kb construct.
3. The activity obtained with Tyr0.5-Luc was variable. In two experiments, the reporter activity obtained with this construct was nearly restored to the levels observed for the full-length promoter. This result could indicate the presence of a negative element situated between –0.5 and –1.1kb upstream of the translation start site. However, in the remaining two experiments the activity was similar to that obtained with Tyr1.1-Luc.
4. Reporter activity as directed by the smallest promoter fragment, Tyr0.248-Luc, was only slightly higher than the activity measured for the promoterless control vector, pGL2-basic. This result is as expected, because important regulatory elements found immediately upstream of this 0.2kb promoter fragment are deleted in Tyr0.248-Luc. Because this deletion significantly reduces transcription, the importance of these elements in mediating transcription initiation is confirmed. Finally, this result also suggests that the promoter fragment in the Tyr0.248-Luc

construct does not contain elements that are able to mediate transcription initiation in melanocytes.

A somewhat different pattern of expression was observed for the deletion constructs in **RPE cells.**

1. The activity obtained with Tyr2.1-Luc was variable. In all but one experiment however, the level of activity measured with this full-length promoter construct was higher than that obtained with any of the shorter constructs.
2. The reporter activity obtained with Tyr1.1-Luc was lower than that obtained with Tyr2.1-Luc. This pattern, which was also observed in melan-a cells, again suggests the presence of a positive element upstream of the 1.1kb promoter fragment.
3. In RPE cells, reporter activity directed by Tyr0.5-Luc was lower than that obtained with the 1.1kb construct. This is in contrast to the result obtained in melan-a cells, where the activity obtained from the 0.5kb construct was equal to or higher than that obtained with the 1.1kb construct. This implies that the negative element upstream of 0.5kb suggested by the melan-a result, is melanocyte specific. Removing this element from the promoter has no effect on RPE cells. This result also suggests that the important promoter elements found in Tyr0.5-Luc are not sufficient to drive high levels of reporter activity in RPE cells. This is in contrast to the result obtained in melan-a cells with Tyr0.5-Luc.
4. Finally, a striking result was obtained with the shortest promoter construct, Tyr0.248-Luc, in RPE cells. This shortest construct consistently yielded higher levels of reporter activity than was obtained from both Tyr0.5-Luc and Tyr1.1-Luc. In one case, the activity driven by Tyr0.248-Luc was equivalent to that obtained with the full-length promoter construct. These results contrast strongly with those obtained with the same promoter construct in melan-a cells. In those cells, the activity obtained with Tyr0.248-luc was the lowest of all the constructs. At face value, this would suggest that the 248bp construct contains elements that mediate the initiation of transcription in RPE cells but not in melanocytes. In addition, the result suggests that the conserved regulatory elements found upstream of the 0.248kb promoter fragment, are not essential for transcription in RPE cells.

In the negative control cell line, **HepG2,** similar, low levels of tyrosinase activity were detected for all constructs.

The above results suggest that the mechanisms that initiate tyrosinase transcription may differ between melanocytes and RPE cells, and even between mammals and avians. However, the interpretation of the interesting result obtained with Tyr0.248-Luc is complicated because the

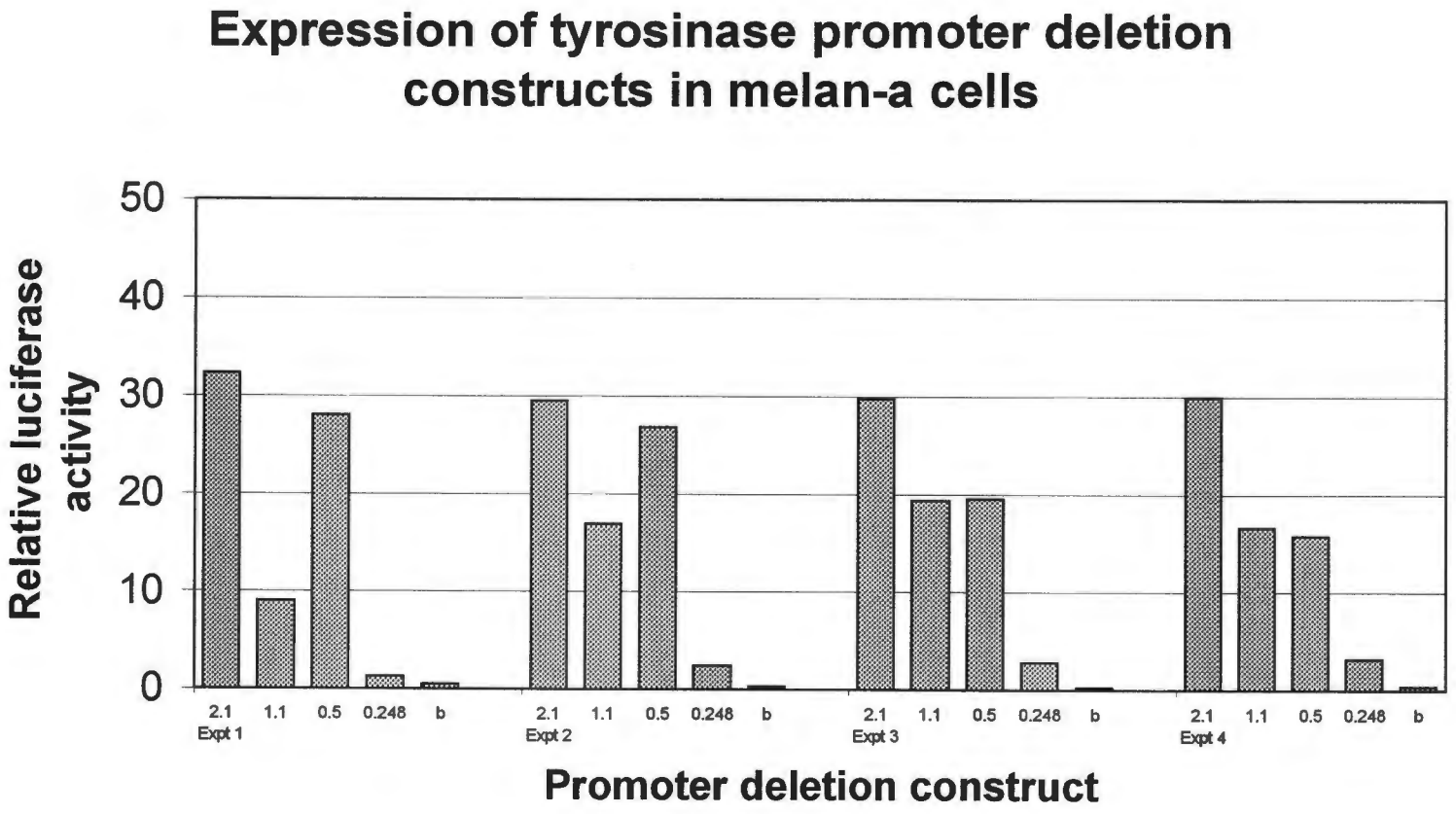


Fig 3.3: Activities of chicken tyrosinase promoter deletion constructs in melan-a cells. Results were normalised with respect to *Renilla* luciferase activity in the same extract and are expressed as a percentage of the luciferase activity obtained with the positive control vector, pGL2-Luc. The figures 2.1, 1.1, 0.5, 0.248 and b on the x-axis represent the constructs Tyr2.1-Luc, Tyr1.1-Luc, Tyr0.5-Luc, Tyr0.248-Luc and pGL2-basic, respectively.

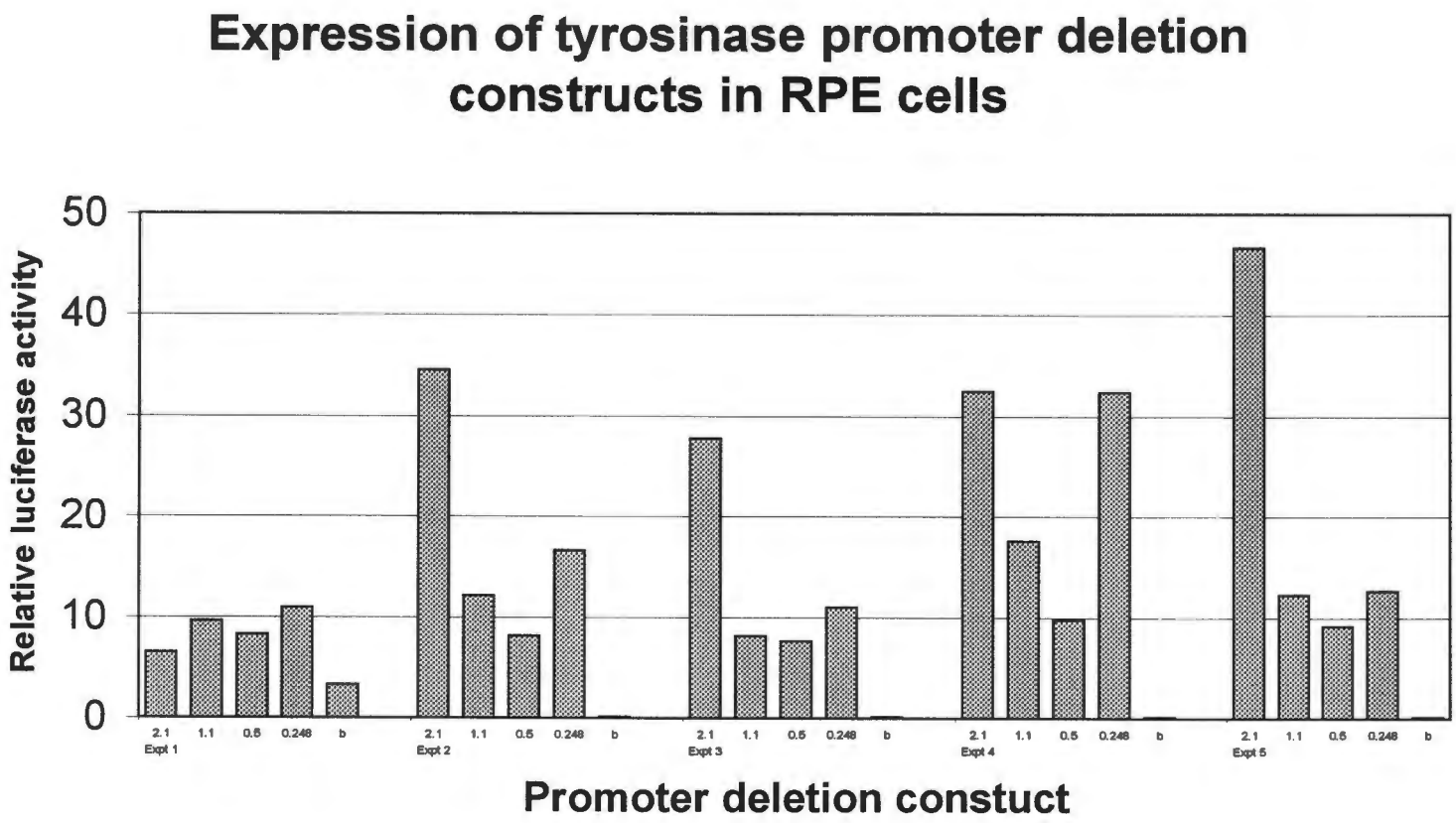


Fig 3.4: Activities of chicken tyrosinase promoter deletion constructs in RPE cells. Results were normalised with respect to *Renilla* luciferase activity in the same extract and are expressed as a percentage of the luciferase activity obtained with the positive control vector, pGL2-Luc. The figures 2.1, 1.1, 0.5, 0.248 and b on the x-axis represent the constructs Tyr2.1-Luc, Tyr1.1-Luc, Tyr0.5-Luc, Tyr0.248-Luc and pGL2-basic, respectively.

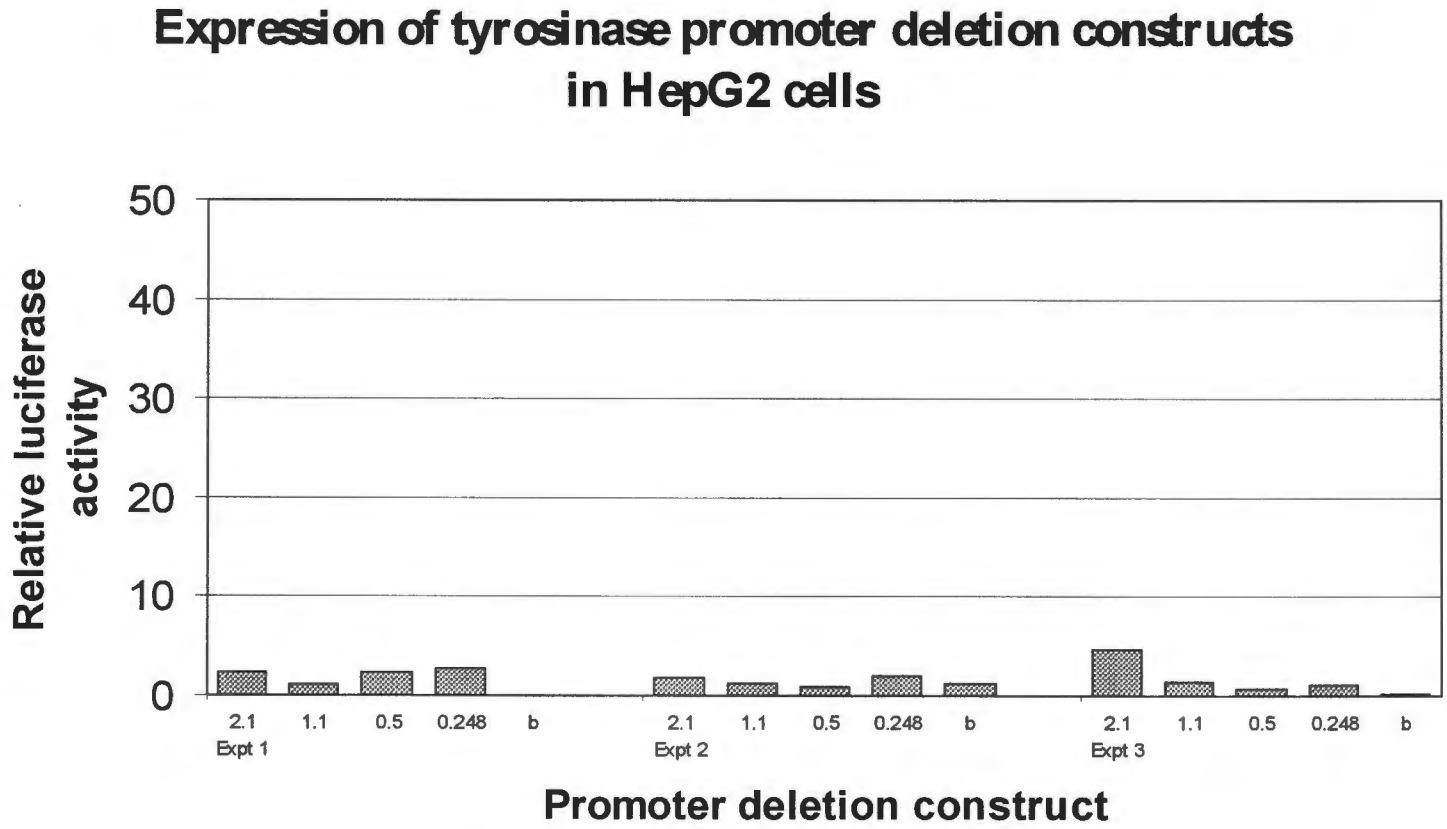


Fig 3.5: Activities of chicken tyrosinase promoter deletion constructs in HepG2 cells. Results were normalised with respect to *Renilla* luciferase activity in the same extract and are expressed as a percentage of the luciferase activity obtained with the positive control vector, pGL2-Luc. The figures 2.1, 1.1, 0.5, 0.248 and b on the x-axis represent the constructs Tyr2.1-Luc, Tyr1.1-Luc, Tyr0.5-Luc, Tyr0.248-Luc and pGL2-basic, respectively.

promoter fragment making up the Tyr0.248-Luc construct consists of two portions of the tyrosinase promoter: 203bp of proximal promoter linked to 45bp from the 5' end of the tyrosinase promoter (as mentioned previously, see Fig 3.2). For this reason, additional experiments were initiated in an attempt to elucidate the mechanism whereby this RPE-specific effect was mediated.

3.5 Cloning of additional tyrosinase promoter deletion constructs.

In order to pinpoint more accurately the location of an element that might mediate the 'RPE-effect' described above, a second series of promoter constructs was cloned (Fig 3.6) for use in transfection experiments in order to address the following specific questions:

1. Is the proximal 203bp promoter alone able to mediate the RPE effect?
2. Alternately, is the 45bp of distal promoter alone able to mediate the RPE effect?
3. Does deletion of the distal 45bp from the full-length (2.1kb) tyrosinase promoter significantly affect tyrosinase expression in RPE cells?

This last question was posed because it seemed unlikely that a distal 45bp promoter fragment would be able to function in isolation, even if it truly was an RPE-specific positive regulatory element. However, it was hoped that its role might be revealed by its deletion from the full-length promoter.

The new promoter deletion constructs were cloned by making use of appropriate restriction enzyme sites present in the tyrosinase promoter sequence and, in some cases, in the vector sequence. The desired promoter fragments were isolated from either Tyr2.1-Luc or from ExoIII 5.25. ExoIII 5.25 is a previously cloned construct consisting of pUC19 into which the full-length tyrosinase promoter was originally cloned (Ferguson and Kidson, 1996). In Tyr2.1-Luc, the restriction site (SmaI) into which the 5' end of the promoter was cloned, was destroyed as a result of the cloning strategy. In ExoIII 5.25, the restriction sites at both the 3'(HindIII) and 5' (EcoRI) ends of the promoter sequence are intact.

Cloning of Tyr0.203-Luc

Tyr0.203-Luc was created to exclude the distal 45bp promoter fragment from Tyr0.248-Luc. This was achieved by isolating the proximal 203bp promoter fragment from the full length tyrosinase promoter in Tyr2.1-Luc, making use of the HindIII site at the 3' end of the promoter and an AsnI site 203bp into the promoter sequence (refer to Fig 2.1 for details). The 203bp fragment was cloned upstream of the luciferase coding region in the promoterless vector, pGL2-basic.

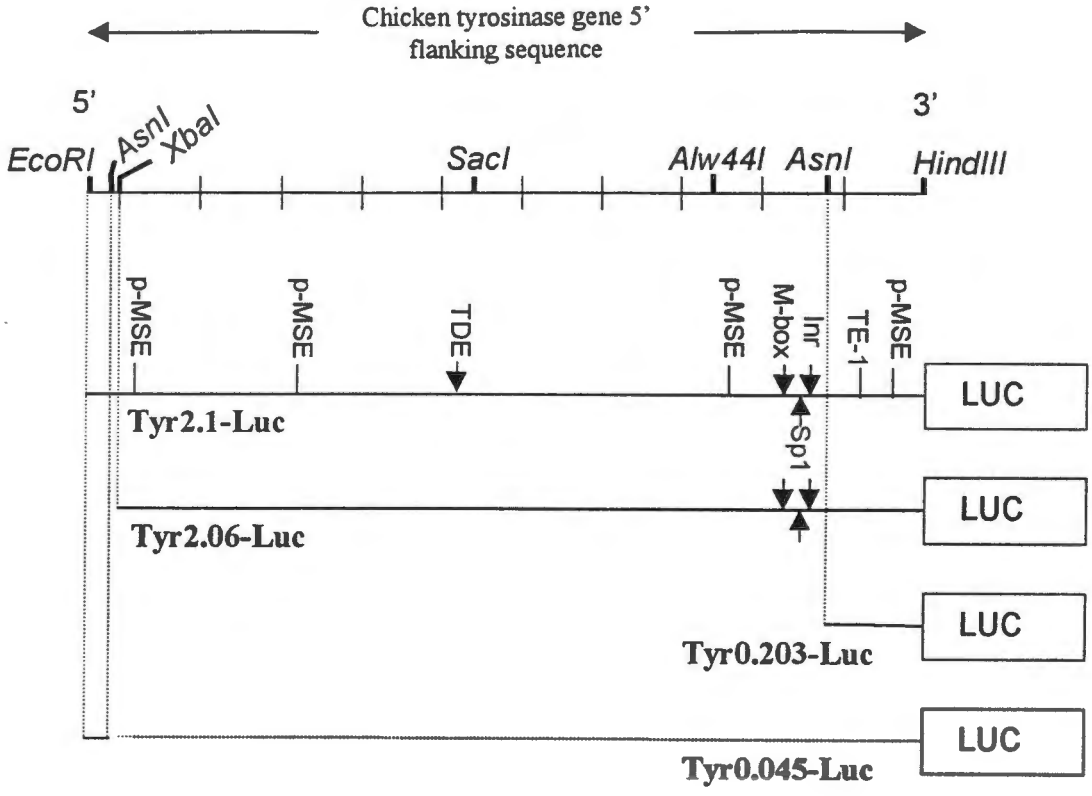


Fig 3.6: Maps of new tyrosinase promoter-luciferase reporter deletion constructs used for additional transient transfection.

Tyr2.06-Luc excludes the distal 63bp of the full-length (2.1kb) promoter. **Tyr0.203-Luc** contains only the proximal 203bp of the full-length promoter. **Tyr0.045-Luc** contains only the distal 45bp of the full-length promoter. (**Tyr2.1-Luc,** which contains the entire 5' flanking sequence cloned to date for the chicken tyrosinase gene, is included as a reference).

Cloning of Tyr0.045-Luc

Tyr0.045-Luc was cloned in order to examine the activity of the 45bp fragment in isolation. Because the restriction site on the immediately 5' of the promoter in Tyr2.1-Luc had been destroyed, the 45bp of 5' vector could not be directly excised from Tyr2.1-Luc. The 45bp fragment could have been isolated from ExoIII 5.25, by digestion with EcoRI and AsnI. However, to avoid working with such a small fragment, Tyr0.045-Luc was prepared as follows (Fig 2.2). Briefly, Tyr2.1-Luc was digested with AsnI and ScaI to release a fragment consisting of 1230bp of 5' vector sequence and 45bp of distal promoter sequence. This fragment was blunt-ended in order to remove the 5' overhang generated by digestion at the AsnI site. The corresponding portion of vector sequence was then excised from pGL2-basic by digestion with ScaI and SmaI. Finally, ligation of the vector-45bp promoter fragment to the truncated pGL2-basic simultaneously restored the reporter vector and introduced the desired promoter fragment upstream of the luciferase coding region.

All the ends of the insert and vector fragments generated as described above, were blunt. Thus, the insert could ligate with the vector in two orientations. However, digestion of the vector at the ScaI site disrupts the vector Ampicillin-resistance cassette. Only if the insert were ligated in the desired orientation would the Amp-resistance cassette be restored. This made it possible to select for the construct with the desired orientation.

Cloning of Tyr2.06-Luc

Tyr2.06-Luc was cloned to specifically exclude the distal 45bp fragment from the full-length tyrosinase promoter. Ideally, this would be done by making use of the AsnI restriction enzyme site situated 45bp downstream of the 5' end of the full-length chicken tyrosinase promoter. A number of strategies were designed which would utilise this AsnI site in order to prepare the desired construct. One such strategy involved the following basic steps:

1. Digestion of ExoIII 5.25 with EcoRI and HindIII to release the full-length tyrosinase promoter.
2. Digestion of the promoter with AsnI, in order to remove the distal 45bp from the full-length sequence.
3. Cloning of the truncated promoter into pGL2-basic.

However, there was a hindrance to the success of any cloning strategy that used the AsnI restriction site. This was the presence of a second AsnI site within the promoter (at 203bp) as well as a number of AsnI sites in the vector sequences (2 sites in pGL2-basic and 3 in pUC19). Therefore, partial restriction digests had to be performed, in order to release a single "full-length minus 45bp" fragment rather than generating two promoter fragments. Although various attempts were made,

the partial restriction digests proved problematic, yielding only very small amounts of the desired fragment. In addition, the cloning strategy outlined above involved multiple steps. At each step, particularly when it was necessary to gel purify a desired fragment, a fraction of the DNA was lost. Thus, large amounts of starting DNA were required in order to obtain sufficient end-product for effective ligation reactions. Many attempts, using a number of variations on the strategy outlined above, were made in the effort to generate a "full-length minus 45bp" promoter construct. Finally however, an alternate cloning strategy was formulated.

The successful cloning strategy made use of a unique XbaI site located 63bp downstream of the 5' end of the full-length tyrosinase promoter. Digestion of the promoter with this enzyme results in a 5' deletion of 63bp from the promoter sequence. This simplified cloning strategy, which avoided the need to perform partial restriction enzyme digests, used ExoIII 5.25 as its starting point (although Tyr2.1-Luc could also have been used). Very briefly, an XbaI/HindIII promoter fragment was isolated from the full-length tyrosinase promoter, and cloned into the SmaI/HindIII sites of pGL2-basic (see Fig 2.3 for details).

The newly cloned constructs were verified by performing diagnostic restriction enzyme digests, as set out in Table 3.2 below. pGL2-basic was included as a positive control to test for the success of each digest, and to serve as a known control vector against which the newly cloned constructs could be compared. For each construct, the expected bands were obtained with the restriction digests.

Table 3.2: Restriction enzymes in diagnostic digests of newly cloned deletion constructs, with expected bands for each new construct and for pGL2-basic.

Construct:	**Tyr0.203-Luc**	**pGL2-basic**
Enzymes used:	HindII	HindII
Bands expected:	1.7 Kb 1.3 kb 1.2 kb 0.353 kb 0.139 kb	2.7 kb 1.3 kb 1.2 kb 0.194 kb 0.139 kb
Construct:	**Tyr0.045-Luc**	**pGL2-basic**
Enzymes used:	HindII + HindIII	HindII + HindIII

Bands expected:	2.7 kb 1.3 kb 1.2 kb 0.239kb 0.139 kb	2.7 kb 1.3 kb 1.2 kb 0.194 kb 0.139 kb
Construct:	Tyr2.06-Luc	**pGL2-basic**
Enzymes used:	HindII	HindII
Bands expected:	3.1 kb 1.8 Kb 1.2 kb 0.408 kb	2.7 kb 1.5 kb 1.2 kb

3.6 Expression of additional tyrosinase promoter deletion constructs in melan-a, RPE and HepG2 cells.

The second series of deletion constructs was cloned in order to address the following question: which portion of the promoter contained in Tyr0.248-Luc was responsible for the high level of reporter activity obtained with this construct in RPE cells? To address this question, transfections were carried out in which melan-a, RPE and HepG2 cells were transfected with the new tyrosinase promoter deletion constructs, Tyr2.06-Luc, Tyr0.203-Luc and Tyr0.045-Luc. In addition to the new constructs, two of the original deletion constructs (the full-length promoter construct, Tyr2.1-Luc, and Tyr0.248-Luc) were included in each experiment to allow comparisons to be made. As before, a *Renilla* luciferase reporter was co-transfected to act as an internal control. Positive and negative controls were included in each experiment. The activity of each new construct was assessed, in duplicate dishes, 3 times for each construct in RPE cells. In melan-a and HepG2 cells, Tyr0.203-Luc was transfected three times while Tyr0.045-Luc and Tyr2.06-Luc were transfected twice each, in duplicate dishes. The results of experiments for the different cell types are presented in Figures 3.7 to 3.9.

In **melan-a** cells, the following results were obtained:

1. The activity obtained with the full-length construct, Tyr2.1-Luc, was similar to that observed in previous experiments.
2. The activity obtained with Tyr0.248-Luc was, as in previous experiments, significantly reduced in comparison to the activity measured with Tyr2.1-Luc.

3. With Tyr2.06-Luc, the reporter activity was variable. In one case, the activity was similar to that measured with the full-length promoter construct. This result implies that the distal 63bp of the chicken tyrosinase promoter do not contain an element that is important for initiating transcription, since deletion of that region appears to have little effect on the level of reporter activity. However, in another experiment, the reporter activity was approximately half that obtained with the full-length promoter.
4. The levels of activity obtained with both Tyr0.203-Luc and Tyr0.045-Luc were similar. That is, in both cases that activity was lower than that obtained with the promoterless control vector, pGL2-basic. This result suggests that neither the 203bp, nor the 45bp promoter fragments are able to mediate transcription initiation.

In **RPE** cells, the pattern of reporter activity was as follows:

1. The reporter activity obtained with the full-length construct was very variable. In two out of three transfections, the reporter activity observed with Tyr2.1-Luc was lower than the activity measured in previous experiments. In the third experiment, the activity was much higher than had previously been measured with this construct.
2. The activity obtained with Tyr0.248-Luc (which was similar to that obtained in previous experiments) was approximately half that measured for Tyr2.1-Luc. This level of reporter activity was still approximately 4 times higher than that measured with the same construct in melan-a cells.
3. The expression measured with Tyr2.06-Luc was again variable. In two instances, the reporter activity level was similar to that obtained with Tyr2.1-Luc. However, in the third experiment, the reporter activity obtained with this construct was more than double the (already high) level of activity measured with Tyr2.1-Luc in the same experiment. This result suggests that the distal 63bp promoter fragment does not contain any essential regulatory elements, as deletion of this region does not, overall, reduce reporter activity in comparison to the full-length promoter.
4. Finally, as in melan-a cells, the reporter activity measured with both Tyr0.203-Luc and Tyr0.045-Luc in RPE cells was only slightly higher than that obtained with pGL2-basic. Once again, this result suggests that neither of these promoter fragments contain elements that are able to initiate transcription.

In **HepG2** cells very low levels of reporter activity were measured, as anticipated. However, the level of activity was not the same for all the constructs. The following pattern of expression was observed:

1. The levels of reporter activity obtained with Tyr2.1-Luc, Tyr0.248-Luc and Tyr2.06-Luc were very similar.
2. The levels of activity obtained with Tyr0.203-Luc and Tyr0.045-Luc, were notably lower than the activity measured with the longer constructs.

The following conclusions can be drawn from the above results:

1. Deletion of the 45bp fragment from Tyr0.248-Luc, to form Tyr0.203-Luc, results in very low levels of reporter activity. The effect of the deletion was most striking in RPE cells, because the activity obtained with Tyr0.248-Luc was unusually high in these cells. This result suggests that the 45bp fragment may contain an element that preferentially activates transcription in RPE cells.
2. The activity obtained with Tyr0.045-Luc was very low in all the cell lines. This result suggests, in contrast to the conclusion in point 1 above, that the 45bp promoter fragment does not contain any regulatory element that is cell-type specific or otherwise.
3. Finally, deletion of the distal 63bp from Tyr2.1-Luc, to form Tyr2.06-Luc, does not significantly reduce transcription in comparison to that measured with the full-length promoter, in any of the cell lines. This result again suggests that the 63bp sequence does not contain any element, tissue specific or otherwise, which is essential for transcription.

The contradicting conclusions outlined above may be reconciled as follows. Neither the 203bp fragment nor the 45bp fragment is able, in isolation, to reproduce the levels of reporter activity obtained with Tyr0.248-Luc (in which the two fragments are linked) in RPE cells. Thus it would appear that the high transcriptional activity obtained with Tyr0.248-Luc is brought about by the juxtaposition of the two fragments in Tyr0.248-Luc, rather than being the result of any regulatory element present in either of the fragments. That is, the RPE effect is mediated by an artificial promoter construct. None the less, it is still interesting that the effect is cell type-specific, being observed consistently in RPE cells but not in melan-a or HepG2 cells. Thus the question arises: how is the 'artificial' promoter sequence in Tyr0.248-Luc able to mediate cell-type specific regulation of reporter activity? Possible answers to this question will be commented on further in Chapter 4.

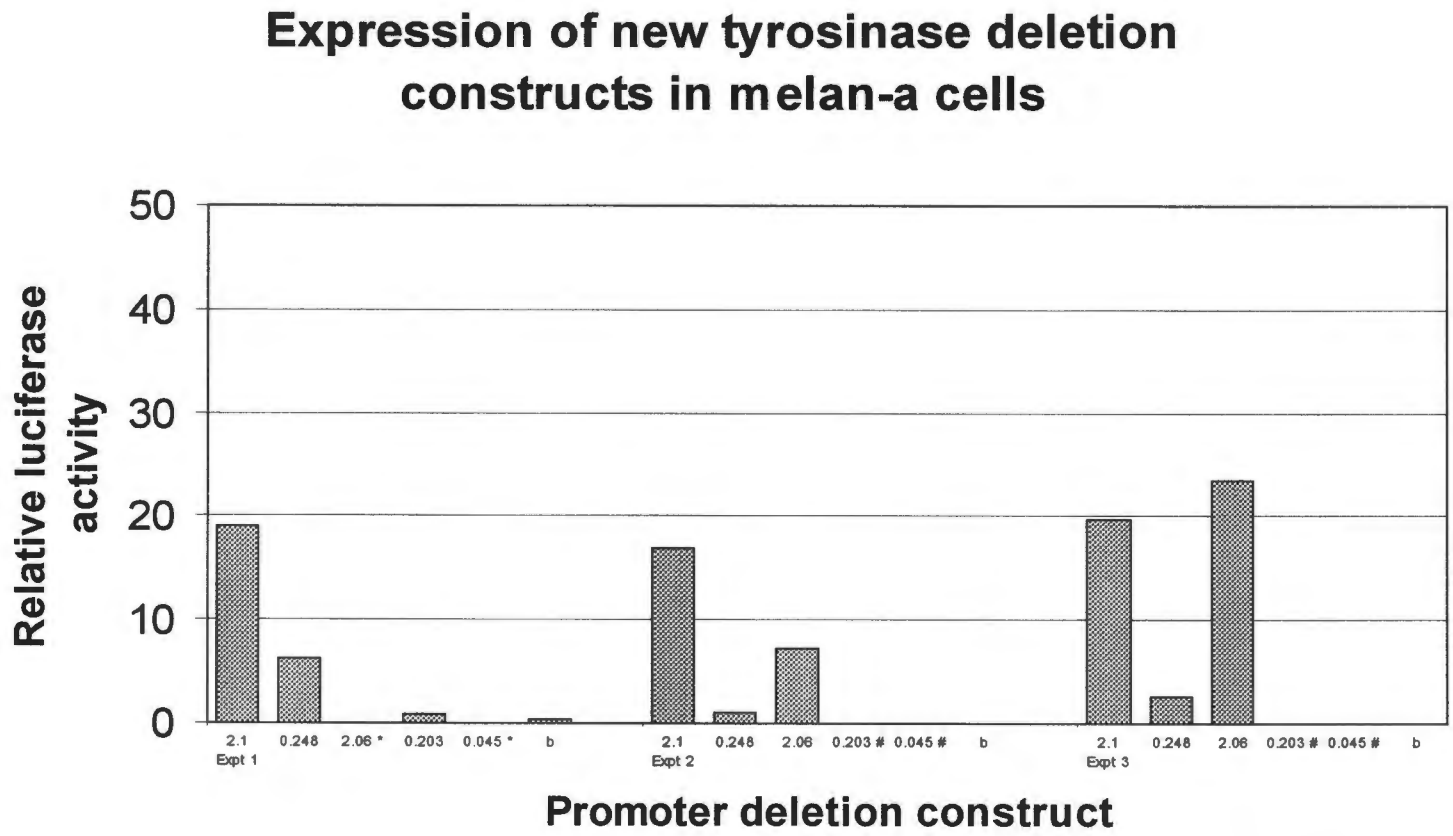


Fig 3.7: Activities of new chicken tyrosinase promoter deletion constructs in melan-a cells. Results were normalised with respect to *Renilla* luciferase activity in the same extract and are expressed as a percentage of the luciferase activity obtained with the positive control vector, pGL2-Luc. The figures 2.1, 0.248, 2.06, 0.203, 0.045 and b on the x-axis represent the constructs Tyr2.1-Luc, Tyr0.248-Luc, Tyr2.06-Luc, Tyr0.203-Luc, Tyr0.045-Luc and pGL2-basic, respectively. A * next to a construct indicates that the construct was not included in a particular experiment. A # next to a construct indicates that the luciferase activity obtained with that construct was too low to be measured.

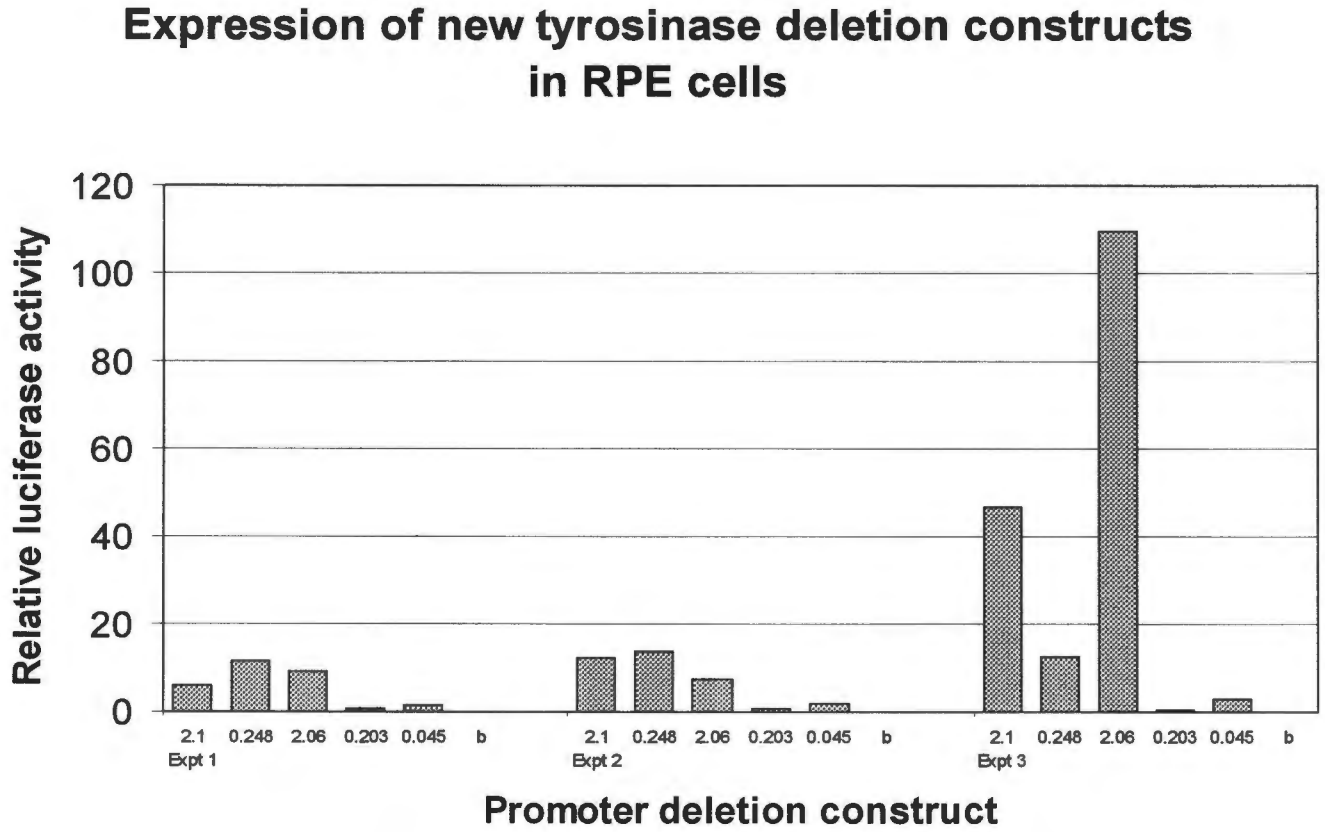


Fig 3.8: Activities of new chicken tyrosinase promoter deletion constructs in RPE cells. Results were normalised with respect to *Renilla* luciferase activity in the same extract and are expressed as a percentage of the luciferase activity obtained with the positive control vector, pGL2-Luc. The figures 2.1, 0.248, 2.06, 0.203, 0.045 and b on the x-axis represent the constructs Tyr2.1-Luc, Tyr0.248-Luc, Tyr2.06-Luc, Tyr0.203-Luc, Tyr0.045-Luc and pGL2-basic, respectively.

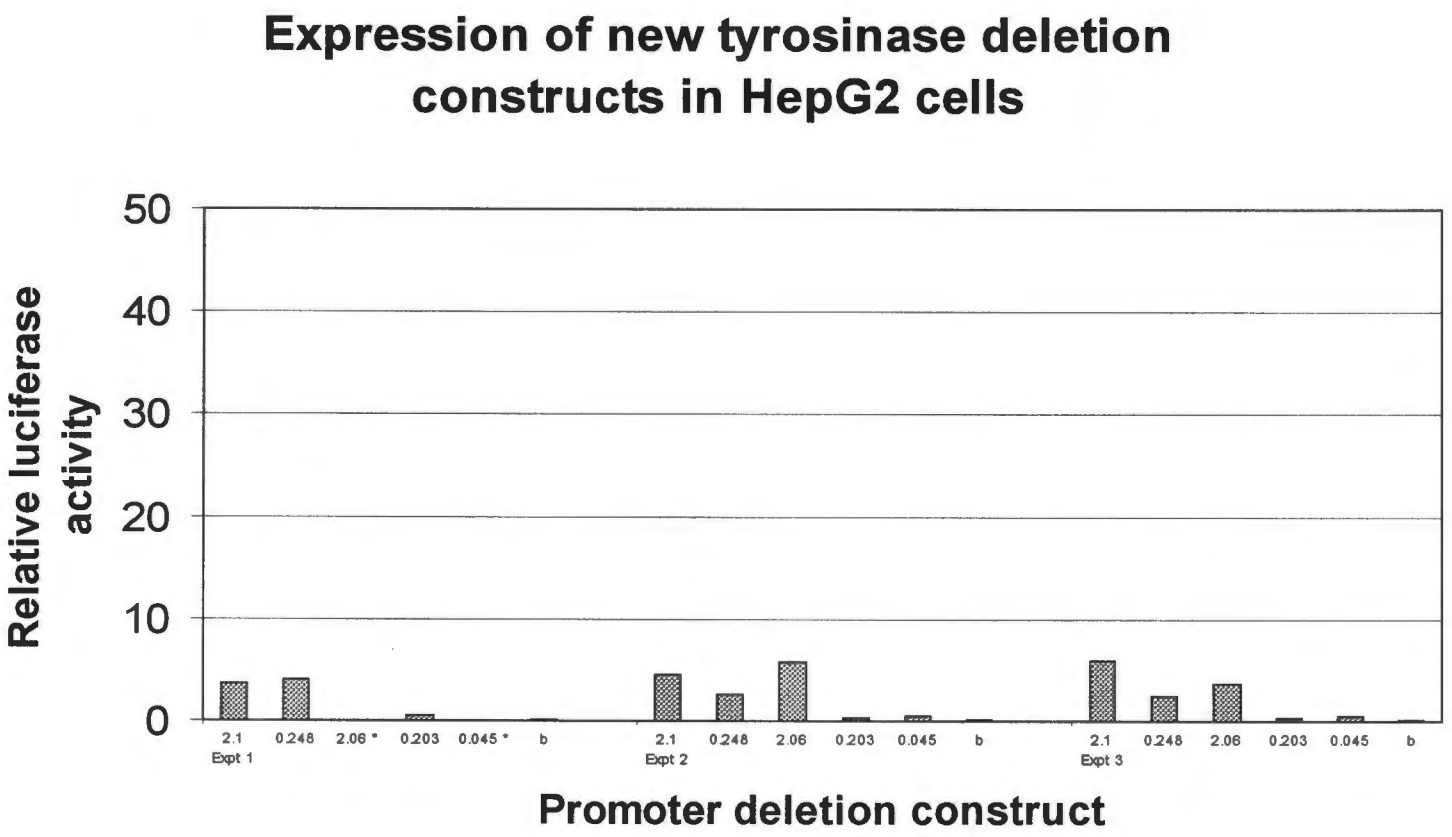


Fig 3.9: Activities of new chicken tyrosinase promoter deletion constructs in HepG2 cells. Results were normalised with respect to *Renilla* luciferase activity in the same extract and are expressed as a percentage of the luciferase activity obtained with the positive control vector, pGL2-Luc. The figures 2.1, 0.248, 2.06, 0.203, 0.045 and b on the x-axis represent the constructs Tyr2.1-Luc, Tyr0.248-Luc, Tyr2.06-Luc, Tyr0.203-Luc, Tyr0.045-Luc and pGL2-basic, respectively. A * next to a construct indicates that the construct was not included in a particular experiment.

CHAPTER FOUR: Discussion

Melanogenesis must be regulated differently in melanocytes and RPE cells: These cell populations have different embryonic origins and differ with respect to the rate at which they produce melanin and the ways in which they respond to melanogenic stimuli. However, very little is known about possible mechanisms that may mediate these differences. The broad aim of the present study was to contribute towards the understanding of the ways in which melanogenesis might be regulated differently in RPE cells and melanocytes. Interesting findings from the present study identify specific regions and elements in the chicken tyrosinase promoter that may have different regulatory roles in melanocytes and RPE cells.

Does the full-length chicken tyrosinase promoter function in a cell type-specific manner?

The first aim of the study was to establish whether the chicken tyrosinase promoter functions in a pigment cell-specific fashion. It was essential to carry out this experiment because the pigment cell-specificity of the chicken tyrosinase promoter had not previously been satisfactorily demonstrated (as described in Chapter 1, section C). To demonstrate that the tyrosinase promoter functions in a tissue-specific manner, it was necessary to demonstrate, at least, that the promoter functions in pigmented cells and not in a non-pigmented cell line. Therefore, in the present study, the full-length tyrosinase promoter construct, Tyr2.1-Luc, was transfected into pigmented melan-a cells (a mouse melanocyte line; Bennett et al, 1987) and chicken RPE cells. The level of reporter activity measured in melanocytes and RPE cells was approximately 6 to 7 times higher than the reporter activity measured in non-pigmented HepG2 cells. These results demonstrate definitively that the chicken tyrosinase promoter is preferentially active in pigmented cells, both melanocytes and RPE cells, over non-pigmented cells. However, the reporter activity obtained with Tyr2.1-Luc was quite weak in comparison to that measured with the positive control vector, pGL2-Luc, in which reporter activity was driven by a strong, constitutively expressed SV40 promoter. This low level of tyrosinase expression may be the result of the long leader sequence found between the putative transcription start point and the luciferase reporter gene (Ferguson and Kidson, 1996). The leader sequence contains at least two ATG triplets that might give rise to abortive translation and diminish the signal from the luciferase reporter. Similarly, the mouse tyrosinase promoter was found to be relatively weak in comparison to a control TK (thymidine kinase) promoter (Klüppel et al, 1991). Klüppel et al did not, however, speculate why this might be so.

Are there differences in the way that the chicken tyrosinase promoter functions in melanocytes and in RPE cells?

To compare promoter activity in melanocytes and RPE cells, a more detailed analysis of the promoter was initiated using a series of promoter deletion constructs in transient transfections experiments. The results of the experiments revealed interesting differences in the way that the promoter behaves in melanocytes and RPE cells.

The results obtained with the deletion construct, **Tyr1.1-Luc,** did not reveal any differences between melanocytes and RPE cells. However, the results shed light on the possible role of a distal tyrosinase promoter element, TDE. The TDE (tyrosinase distal element), which was first identified in the human tyrosinase promoter, contains a central E-box motif (CATGTG) and was found to possess tissue-specific enhancer activity in transient transfection assays (Yasumoto et al, 1994). In the present study, melanocytes and RPE cells transfected with Tyr1.1-Luc, a deletion construct that excludes a TDE-like sequence, showed a decrease in reporter activity compared to the activity obtained with Tyr2.1-Luc. This was true for both melanocytes and RPE cells. This result is in agreement with the pilot transfection experiments carried out by Ferguson (1996, unpublished), and suggests that, as in the human, the chick TDE-like element acts as a transcriptional enhancer element.

In contrast to the above results, analysis of promoter activity obtained with **Tyr0.5-Luc** did reveal differences in activity between melanocytes and RPE cells. Tyr0.5-Luc contains the core promoter elements (the initiator region, the Sp1-binding site and the highly conserved M-box) of the proximal conserved region of the chicken tyrosinase promoter, and excludes the TDE. Reporter activity in melanocytes transfected with Tyr0.5-Luc was similar to that obtained with the full-length promoter construct, Tyr2.1-Luc. This result demonstrates that, in melanocytes, the core elements alone are sufficient to direct high levels of reporter activity. In contrast, in RPE cells the reporter activity obtained with Tyr0.5-Luc was significantly lower than the activity obtained with Tyr2.1-Luc. Thus, it would appear that in RPE cells, the core promoter elements contained in Tyr0.5-Luc are not sufficient to direct levels of reporter activity similar to those obtained with the full-length promoter. This might suggest that RPE cells have a greater requirement than melanocytes for the enhancer activity of the TDE. Functional studies of the TDE in mammals have only been carried out in melanoma cell lines and no information is available regarding the functional significance of the TDE in RPE cells. The results of the present study suggest that the role of the proximal conserved elements and of the TDE is different in RPE cells and melanocytes.

In addition to suggesting different roles for the conserved elements and the TDE in RPE cells and melanocytes, the different pattern of Tyr0.5-Luc expression in these cells suggests the presence of at least one other cell type-specific regulatory element in the chicken tyrosinase promoter. In melanocytes, the activity obtained with Tyr0.5-Luc was higher than the activity obtained with the longer construct, Tyl.1-Luc. This result suggests the presence of a negative regulatory element between -0.5 and -1.1kb from the ATG. Deletion of this sequence would relieve negative regulation and allow high levels of transcription to be initiated from the core promoter elements. Such a negative regulatory element was reported in both the human and mouse tyrosinase promoters (Bentley et al, 1994; Ganss et al, 1994). In both these cases, the element was located just upstream of the conserved M-box. If there is indeed a similar element in the chicken tyrosinase promoter, and if it is located in the same position relative to the M-box, it is likely to be included in Tyr0.5-Luc, rather than deleted. However, the proximal conserved region identified in the chicken tyrosinase promoter (Ferguson and Kidson, 1996) ends just upstream of the M-box. Therefore, it is possible that in the chicken gene, the negative regulatory element is further upstream than in the mouse and human tyrosinase promoters (see Fig 4.1). If this were so, the negative element would be deleted from the sequence contained in Tyr0.5-Luc. Deletion of this element would explain why, in melanocytes, a higher level of reporter activity was obtained with Tyr0.5-Luc than with Tyr1.1-Luc.

In contrast to melanocytes, in RPE cells the reporter activity obtained with Tyr0.5-Luc was similar to the low level obtained with Tyr1.1-Luc. Thus, if there is indeed a negative element responsible for the expression pattern in melanocytes, it appears that it exerts a melanocyte-specific regulatory effect. This is consistent with the report of Ganss et al (1994). They found that mutation of the negative element upstream of the M-box in the mouse tyrosinase promoter resulted in increased expression in melanocytes but not in a non-pigmented control cell line. However, their study did not investigate the functional significance of the negative element in RPE cells. Ganss et al (1994) propose that, in melanocytes, the induction of tyrosinase expression by external factors such as UV or α-MSH may be mediated by the removal of a repressor binding to the negative regulatory element. RPE cells are apparently unresponsive to such external stimuli, and it is interesting to speculate that this is because the negative promoter element identified by Ganss et al (1994) is not functional in RPE cells. Further studies would be useful in clarifying this point.

The results of transfections of the shortest promoter construct from the original deletion series, **Tyr0.248-Luc,** again revealed interesting differences in tyrosinase activity in melanocytes and RPE cells. Tyr0.248-Luc contains no elements known to regulate tyrosinase transcription in other

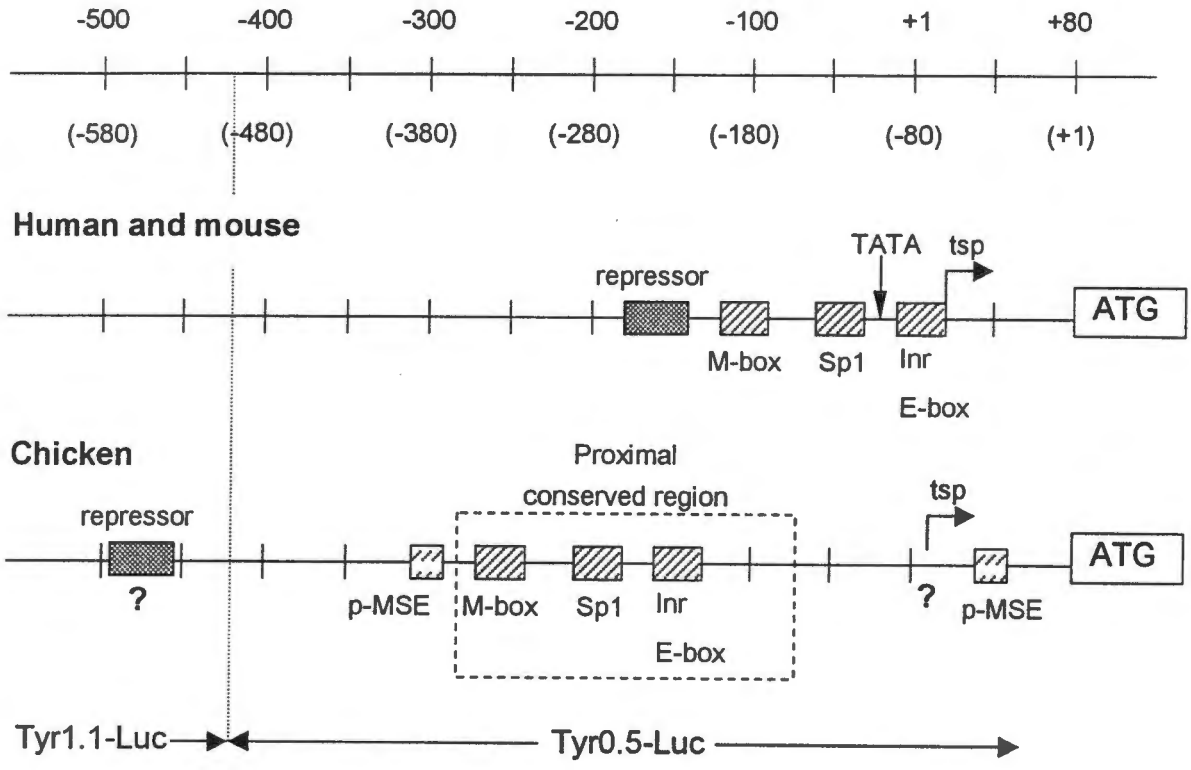


Fig4.1: Possible position of negative regulatory element in the mammalian (human and mouse) and avian (quail and chicken) tyrosinase promoters.

The chicken tyrosinase promoter deletion construct, Tyr0.5-Luc, possibly excludes the negative (repressor) element found in the equivalent mouse and human promoter fragment, because the element lies outside and hence, perhaps, further upstream from, the proximal conserved region of the chicken promoter. This would account for the higher level of reporter activity obtained with Tyr1.1-Luc than with the shorter Tyr0.5-Luc, in melanocytes (see text for details).

animals. As expected, the reporter activity measured in melanocytes transfected with Tyr0.248-Luc was very low. This result confirms the importance for melanocytes, of the conserved core elements (the Inr, Sp1 site and M-box) located upstream of the 0.2kb promoter fragment. In addition, the result suggests that this 248bp promoter construct does not contain any elements that are able to initiate transcription in melanocytes. In striking contrast to the result obtained in melanocytes, the reporter activity obtained with Tyr0.248-Luc in RPE cells was consistently higher than the activity obtained with both Tyr0.5-Luc and Tyr1.1-Luc. In one instance, the level of reporter activity obtained with Tyr0.248-Luc was equivalent to that obtained with the full-length construct in these cells. Similar results to these were first obtained by Ferguson (1996, unpublished data), who found the activity obtained with Tyr0.248-Luc in RPE cells to be higher than that obtained with the full-length construct, Tyr2.1-Luc. These results are particularly striking given that Tyr0.248-Luc contains none of the elements known to mediate even basal transcription initiation, much less an RPE-specific activity. To distinguish between possible explanations for this result and in an attempt to determine more accurately the location and possible nature of the elements responsible for mediating the RPE-specific effect, additional deletion constructs of the chicken tyrosinase promoter were made and transfected into melanocytes and RPE cells.

What portion of the promoter fragment contained in Tyr0.248-Luc contains elements able to direct high levels of reporter activity in RPE cells?

Tyr0.203-Luc was constructed to contain only the proximal 203bp of the chicken tyrosinase promoter (see Fig 3.6). The reporter activity observed with this construct in RPE cells was greatly reduced in comparison to the activity obtained with Tyr0.248-Luc, which carries the distal 45bp promoter fragment. This result indicates that the proximal 203bp promoter fragment does not allow transcription initiation and suggests that the distal 45bp fragment contains the element responsible for the high levels of reporter activity measured with Tyr0.248-Luc. However, the reporter activity obtained with the 45bp tyrosinase fragment alone **(in Tyr0.045-Luc)** remained reduced in comparison to that obtained with Tyr0.248-Luc. This result indicates that the 45bp fragment is not, in isolation, able to mediate high levels of tyrosinase expression. In addition, deletion of the distal 63bp (including the 45bp in question) to form **Tyr2.06-Luc**, did not result in reduced reporter gene expression in comparison to that obtained with the full-length promoter sequence. This result suggests that the 45bp fragment does not contain an enhancer element responsible for the high level of reporter activity obtained with Tyr0.248-Luc.

Taken together, the results described above indicate that neither the proximal 203bp nor the distal 45bp promoter fragments are able, in isolation, to reproduce the high levels of reporter activity

obtained in RPE cells when the two fragments are combined. Thus, it is now clear that the RPE-specific effect observed with Tyr0.248-Luc is the result of the *combination* of the 203 and 45bp promoter regions. In short, the high level of reporter activity obtained in RPE cells with Tyr0.248-Luc is driven by an artefactual promoter construct. However, interesting questions remain to be answered. For example, how is this artefactual promoter able to mediate transcription in RPE cells and not in melanocytes? What possible elements could be contained in the promoter sequences of Tyr0.248-Luc to mediate the RPE-specific effect? This question was first addressed by Ferguson (1996, unpublished data), who suggests that the 203bp proximal promoter sequence has the potential to initiate basal levels of transcription. The following characteristics of the promoter fragment led Ferguson to this conclusion. Firstly, the sequence in question contains a potential Inr region that overlaps the transcription start site reported for the quail tyrosinase gene. This is located just upstream of a transcription start site identified for the chicken tyrosinase gene. Secondly, the sequence contains homologues of two quail tyrosinase elements (TE-1 and a proximal p-MSE) that are thought to have enhancer activity, although their functional significance in the quail is not known. Together, these elements may allow for transcription initiation from the promoter fragment. In order to account for the very high level of activity obtained with Tyr0.248-Luc in RPE cells, Ferguson suggests that the 45bp fragment may contribute an RPE-specific enhancer element to the promoter construct. However, no such element of apparent significance to RPE-specific gene regulation was identified (Ferguson, 1996, unpublished data).

A further complicating factor in the interpretation of the present results is that both avian and mammalian cell lines were used in the present analysis. That is, the results obtained in a *mouse* melanocyte cell line were compared with those obtained in *chicken* RPE cells. This system was necessary because of the lack of suitable chick melanocyte cell lines in which to carry out the experiments. Other studies of tyrosinase expression have used such mixed systems. For example, the human tyrosinase promoter was shown to be efficiently expressed in the mouse melanoma cell line, B16 (Bentley et al, 1994). Also, both the quail and mouse tyrosinase promoters have been shown to direct pigment cell-specific expression of a downstream tyrosinase cDNA in cultured chicken neural crest cells (Akiyama et al, 1994). The accurate cross-species activity of the tyrosinase promoter in these experiments was perhaps to be expected, given the high degree of conservation of the promoter between species. None the less, the avian tyrosinase promoter (both quail and chick) does differ in certain respects from the mammalian promoters.

It is thus possible that, in the present study, some of the differences observed between melanocytes and RPE cells could be the result of differences between tyrosinase expression in mammals and

avians, rather than between melanocytes and RPE cells. In particular, it is possible that the surprising result obtained with Tyr0.248-Luc in RPE cells came about because the RPE cells are avian cells, and thus contain certain factors that recognise the avian-specific elements in the Tyr0.248-Luc promoter fragment. The mouse melanocytes on the other hand, would not contain the appropriate factors and this would explain why expression from Tyr0.248-Luc was low in these cells, compared to RPE cells. None the less, the possibility cannot be excluded that an RPE-specific element (rather than simply an avian-specific element) is present in the promoter sequences contained in Tyr0.248-Luc. This is because very little is known about what constitutes an RPE specific element.

How is the expression of genes in the RPE regulated?

Some information concerning the mechanisms whereby gene expression is regulated in the RPE can be gleaned from studies of other genes differentially expressed in the retinal pigment epithelium. Only two non-melanogenic genes that are expressed in the RPE have been characterised with respect to their 5' flanking regions.

Cellular retinaldehyde-binding protein (CRALBP) is abundantly expressed in the RPE and in Müller cells of the retina. It is a substrate carrier in the visual cycle and plays a role in regeneration of photoreceptor visual pigment. In addition, the CRALBP gene has been implicated in the degenerative eye disease, retinitis pigmentosa. In order to elucidate the regulatory regions required for RPE-specific expression of the gene, the promoter of the CRALBP gene was cloned and characterised (Kennedy et al, 1998). Promoter analysis of the CRALBP gene revealed the presence of two photoreceptor consensus element-1 (PCE-1) sites in the proximal 250bp of promoter. The PCE-1 motif (CAATTAG) has been proposed to direct photoreceptor-specific gene expression. However, transient transfection and gel mobility shift assays in RPE cells have now shown that mutation or deletion of either PCE-1 site in the CRALBP promoter significantly decreases promoter activity and reduces protein binding to the promoter. Thus, the PCE-1 motif and binding factors may play an important role in the regulation of genes in RPE cells as well as photoreceptor cells (Kennedy et al, 1998).

RPE65 is a 61 kDa protein that is expressed specifically in the RPE. Its function is not yet known, but it is proposed to play a role in the RPE/photoreceptor vitamin A cycle. It too has been implicated in retinal disease, and recent studies have focussed on understanding the regulation of RPE65 transcription. In their analysis of the RPE65 5' flanking regions, Nicoletti et al (1998) identified sequences homologous to the CRALBP gene. In DNase I Footprint assays they identified

a number of protected regions, which included sequences for an E-box motif and a PCE-1-like motif. This result again suggests that the PCE-1 motif may be important in the regulation of gene expression in the RPE. However, the regions containing these elements were protected in nuclear extracts from both HeLa cells and a human RPE cell line and are thus likely to be responsible for basal rather than RPE-specific promoter activity. Ultimately, Nicoletti et al (1998) conclude that additional, as yet unidentified, regulatory elements are likely to be involved in restricting gene expression to the retinal pigment epithelium.

Interestingly, examination of the promoter sequence contained in Tyr0.248-Luc has revealed the presence of a PCE-1-like element that is formed at the junction of the 203 and 45bp fragments. This element, which is present in the complementary strand of the promoter sequence, has a 6/7 match with the PCE-1 consensus sequence. It is interesting to speculate that PCE-1 binding factors in RPE cells may interact with this element in Tyr0.248-Luc to activate transcription from the basal promoter elements further downstream. The absence of such factors in melanocytes would account for the low levels of expression obtained with Tyr0.248-Luc in those cells. In addition, because both the distal 45bp and the proximal 203bp promoter fragments contribute a portion of the sequence that creates the PCE-1-like motif, this could explain why neither of the fragments in isolation is able to initiate reporter activity.

RPE-specific gene regulation by Tyrosinase family member genes

Other genes obviously worthy of attention for gaining insight into RPE-specific expression are the tyrosinase gene family members, Trp-1 and DCT, which are expressed primarily in melanocytes and the RPE. Both of these genes are fairly well characterised with respect to their promoter functions. In addition, because of the high degree of conservation amongst the family members, it seems possible that similar mechanisms will regulate their expression. However, careful comparison has revealed a greater homology between the Trp-1 and DCT genes than between Tyr and the two related proteins (Jackson et al, 1991; Morrison et al, 1994; Sturm et al, 1995). In addition, an increasing amount of evidence now points to differences in the regulation of promoter activity of the three genes. Some recent work highlighting such differences is mentioned below.

Firstly, Carreira et al (1998) point out that the tyrosinase and Trp-1 promoters contain only a single common element, the M-box. Secondly, the genes interact differently with microphthalmia-associated transcription factor, Mitf. A study by Smith et al, 1998, has provided in vivo evidence that Mitf regulates both Trp-1 and tyrosinase gene transcription. However, the human DCT promoter has alternately been shown unresponsive to Mitf, (Yasumoto et al, 1997), or to be strongly

stimulated by Mitf binding to the conserved M-box (Bertolotto et al, 1998). Thirdly, Carreira et al (1998) have identified the brachyury-related transcription factor Tbx2 as a repressor of Trp-1, but not tyrosinase, promoter activity.

The above-mentioned studies focussed almost exclusively on promoter activity in melanocyte or melanoma cells lines. However, Raymond and Jackson (1995) found that a LacZ reporter gene driven by various Trp-1 promoter fragments in transgenic mice was expressed strongly in the RPE but very poorly, if at all, in neural crest derived melanocytes. Likewise, Schmidt et al (1998) used a 4kb Trp-1 promoter fragment to direct expression of a RET oncogene to the RPE of transgenic mice. It is interesting that Trp-1 promoter activity in these transgenics should be limited to the RPE, given that Trp-1 is in fact expressed in all pigment cells. Is it possible that an as-yet-uncharacterised property of the Trp-1 promoter is involved in differential expression of Trp-1 in the RPE and melanocytes? Unfortunately, no work has yet shown this the case. In addition, there is an increasing body of evidence that indicates that the tyrosinase gene family members are regulated differently. Therefore, it would be necessary to exercise caution in extrapolating an understanding of RPE-specific regulation of Trp-1 to that of tyrosinase.

APPENDIX I: Solutions used

TPA stock and working solutions

A 2mM stock solution was prepared by resuspending the contents of a 10mg vial of TPA (12-O-tetradecanoyl phorbol-13-acetate, MW = 616.8) in 8.11ml of absolute ethanol. Aliquots were stored in foil-covered eppendorfs at –80°C.

A 40µM working solution was prepared by diluting 200µl of the stock solution with 9800µl of sterile phosphate buffered saline (PBS) containing 1mg/ml bovine serum albumen (BSA). Aliquots were stored in foil-covered eppendorfs at –80°C.

Penicillin and Streptomycin

A stock solution of 10 000 I.U./ml each of penicillin and streptomycin was prepared by dissolving 0.6g of penicillin (1662 I.U./mg) and 1.335g of streptomycin (750 I.U./mg) in 100ml distilled water. The solution was sterilised by filtration through a 0.22µm filter. Aliquots were stored at –20°C.

CMF Hanks

For 1L:

0.4g KCl

0.06g KH_2PO_4

8g NaCl

0.35g $NaHCO_3$

0.48g NaH_2PO_4

Make up to 900ml in dH_2O, adjust pH to 7.4, make up to 1L, autoclave and store at 4°C.

Glucose buffer

25mM Tris, pH 8.0

50mM glucose

10mM EDTA, pH8.0

NaOH/SDS

0.2M NaOH with 1% SDS – prepared fresh each time by mixing 5ml of 10% SDS, 44ml of dH_2O and 1ml of 10M NaOH.

Potassium acetate solution

For 100ml:

29.4g potassium acetate

11.5ml glacial acetic acid

make up to 100ml with dH_2O

2 x HBS

10g/L Hepes

16g/L NaCl

Adjust pH to 7.10 (+/- 0.05)

Autoclave

Store at room temperature for no longer than one month.

2M $CaCl_2$

For 50ml dissolve 14.7g $CaCl_2 \cdot 2H_2O$ in 40ml dH_2O, make up to 50ml with dH_2O, sterile filter through a 0.22µm filter. Store at room temperature.

100 x PO_4

For 100ml:

1.25g $Na_2HPO_4 \cdot 2H_2O$

1.09g $NaH_2PO_4 \cdot 2H_2O$

Make up to 100ml in dH_2O, autoclave.

Store at room temperature.

ß-gal assay solution 1

60mM Na_2HPO_4

40mM NaH_2PO_4

10mM KCl

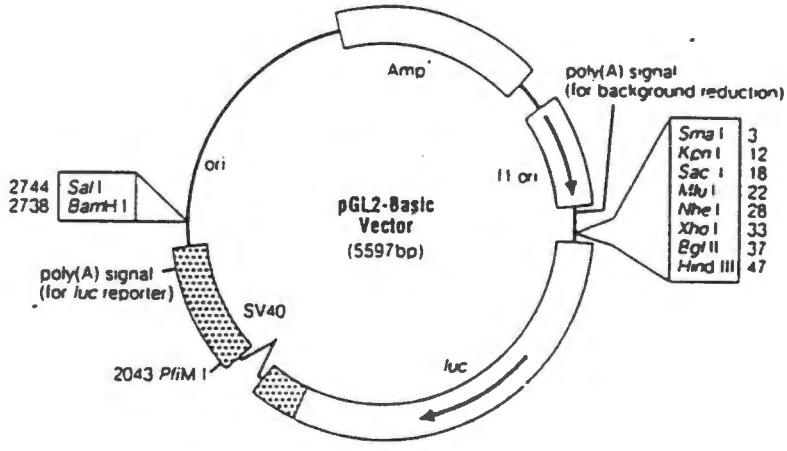
1mM $MgCl_2 \cdot 6H_2O$

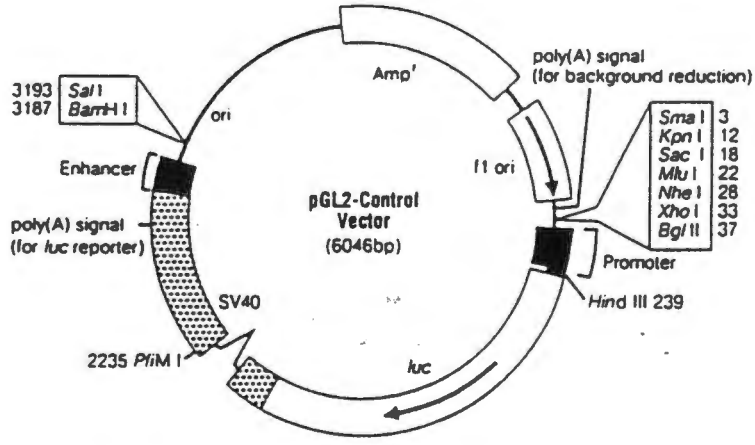
50mM β-mercaptoethanol

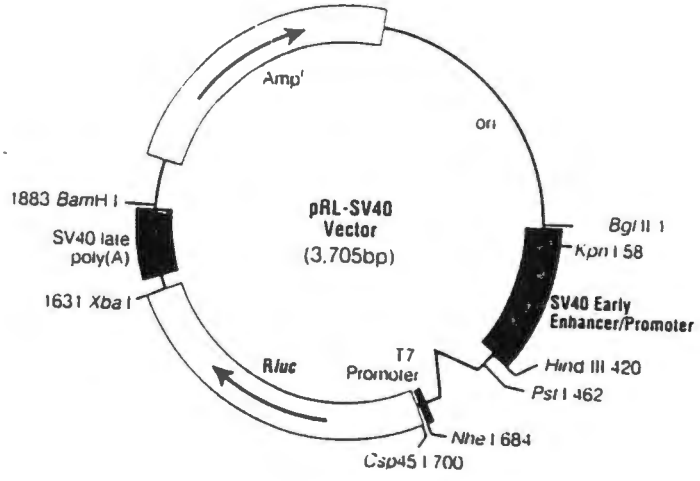
ß-gal assay solution 2

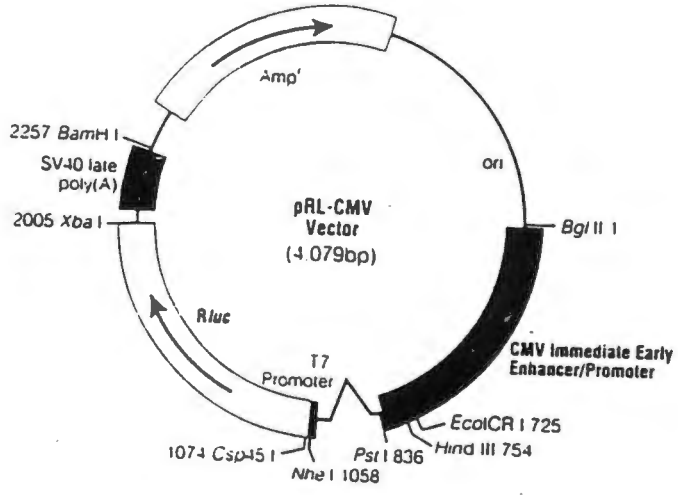
2mg/ml ONPG in 60mM Na_2HPO_4 and 40mM NaH_2PO_4.

APPENDIX II: Vectors used






Amp'
ori
1883 BamH I
SV40 late poly(A)
1631 Xba I
pRL-SV40 Vector (3,705bp)
Bgl II 1
Kpn I 58
SV40 Early Enhancer/Promoter
Hind III 420
Pst I 462
T7 Promoter
Rluc
Nhe I 684
Csp45 I 700


Amp'
ori
2257 BamH I
SV40 late poly(A)
2005 Xba I
pRL-CMV Vector (4,079bp)
Bgl II 1
Rluc
T7 Promoter
CMV Immediate Early Enhancer/Promoter
EcoICR I 725
Hind III 754
Pst I 836
1074 Csp45 I
Nhe I 1058

APPENDIX III: Additional results

Table 1: Transfection of melan-a cells with pRSV-βgal using FuGENE 6 transfection reagent.

Treatment of cells			β-galactosidase activity measured (OD_{420}) *	Morphology of melan-a cells
	FuGENE 6 added	pRSV-βgal plasmid added		
Experimental cells	✓	✓	0.545	Normal morphology
'Mock transfected' cells	✓	✗	0.252	Normal morphology
Untreated control cells	✗	✗	0.275	Normal morphology

* The β-galactosidase assay was allowed to proceed overnight.

Table 2: Transfection of melan-a cells with pGL2-luc and Tyr2.1-Luc using FuGENE 6 transfection reagent.

	Dish number	Plasmid added		Light units measured
		pGL2-luc	Tyr2.1-Luc	
Experiment 1 (to confirm that transfection was taking place)	**1.1**	✓	✗	0.365
	control	✗	✗	0.000
Experiment 2 (to determine if measurable reporter activity is obtained with Tyr2.1-Luc)	**2.1**	✓	✗	0.034
	2.2	✗	✓	0.014
	control	✗	✗	0.000
Experiment 3 (to determine the optimum ratio of DNA : FuGENE 6)	**3.1** (2:3)*	✓	✗	0.223
	3.2 (1:2)	✓	✗	0.096
	3.3 (1:3)	✓	✗	0.039
	3.4 (1:6)	✓	✗	0.095
	control	✗	✗	0.000

*Numbers in brackets indicate the ratio of DNA (μg) : FuGENE 6 (μl). Where not indicated, a ratio of 2:3 was used.

Fig 1: Activity of the full-length chicken tyrosinase promoter construct, Tyr2.1-Luc, in melan-a cells. The experiment was repeated 11 times. Results were normalised with respect to *Renilla* luciferase activity in the same extract and are expressed as a percentage of the luciferase activity obtained with the positive control vector, pGL2-Luc. The figures 2.1 and basic on the x-axis represent the constructs Tyr2.1-Luc and pGL2-basic, respectively.

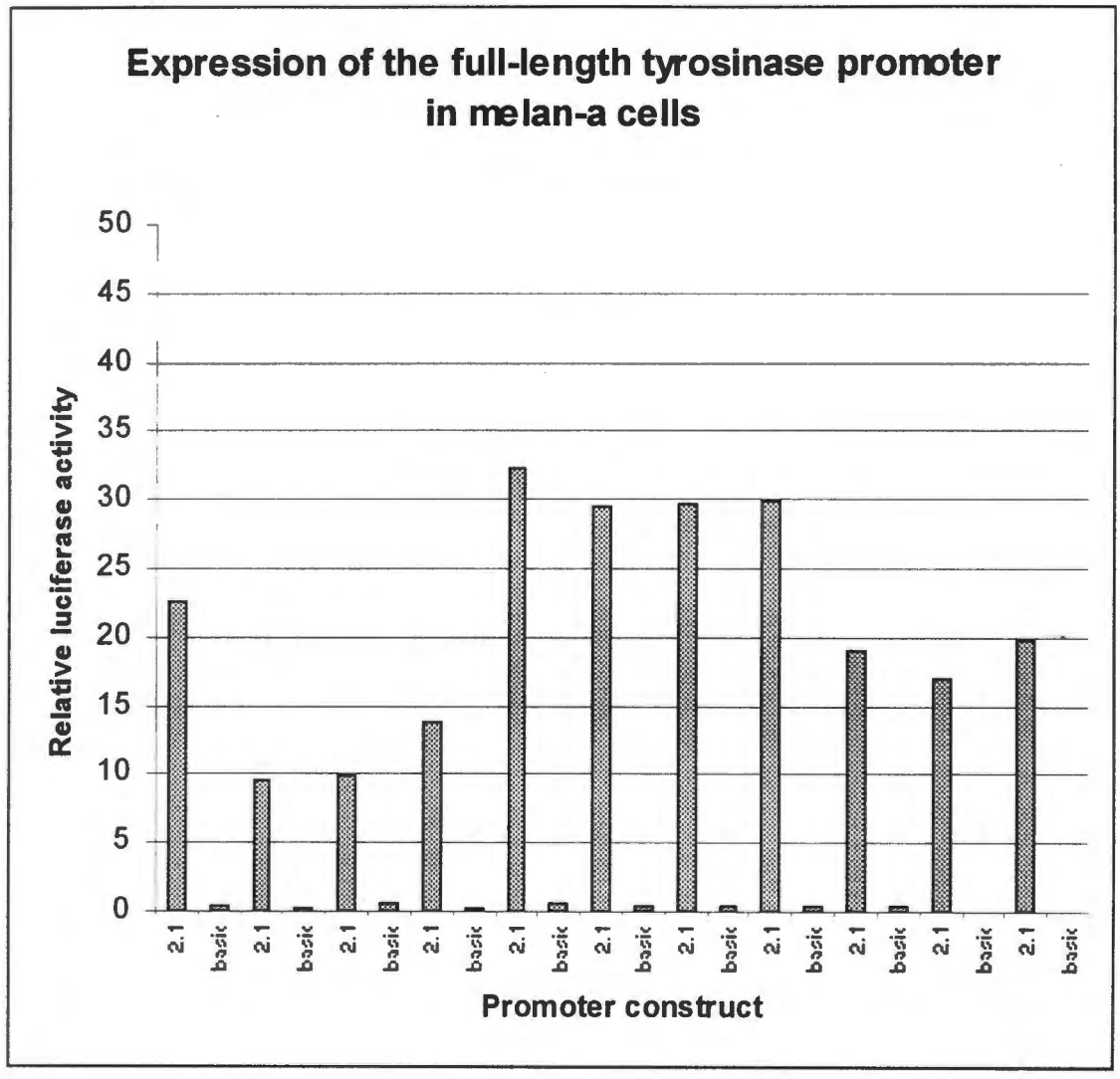


Fig 2: Activity of the full-length chicken tyrosinase promoter construct, Tyr2.1-Luc, in RPE cells. The experiment was repeated 9 times. Results were normalised with respect to *Renilla* luciferase activity in the same extract and are expressed as a percentage of the luciferase activity obtained with the positive control vector, pGL2-Luc. The figures 2.1 and basic on the x-axis represent the constructs Tyr2.1-Luc and pGL2-basic, respectively.

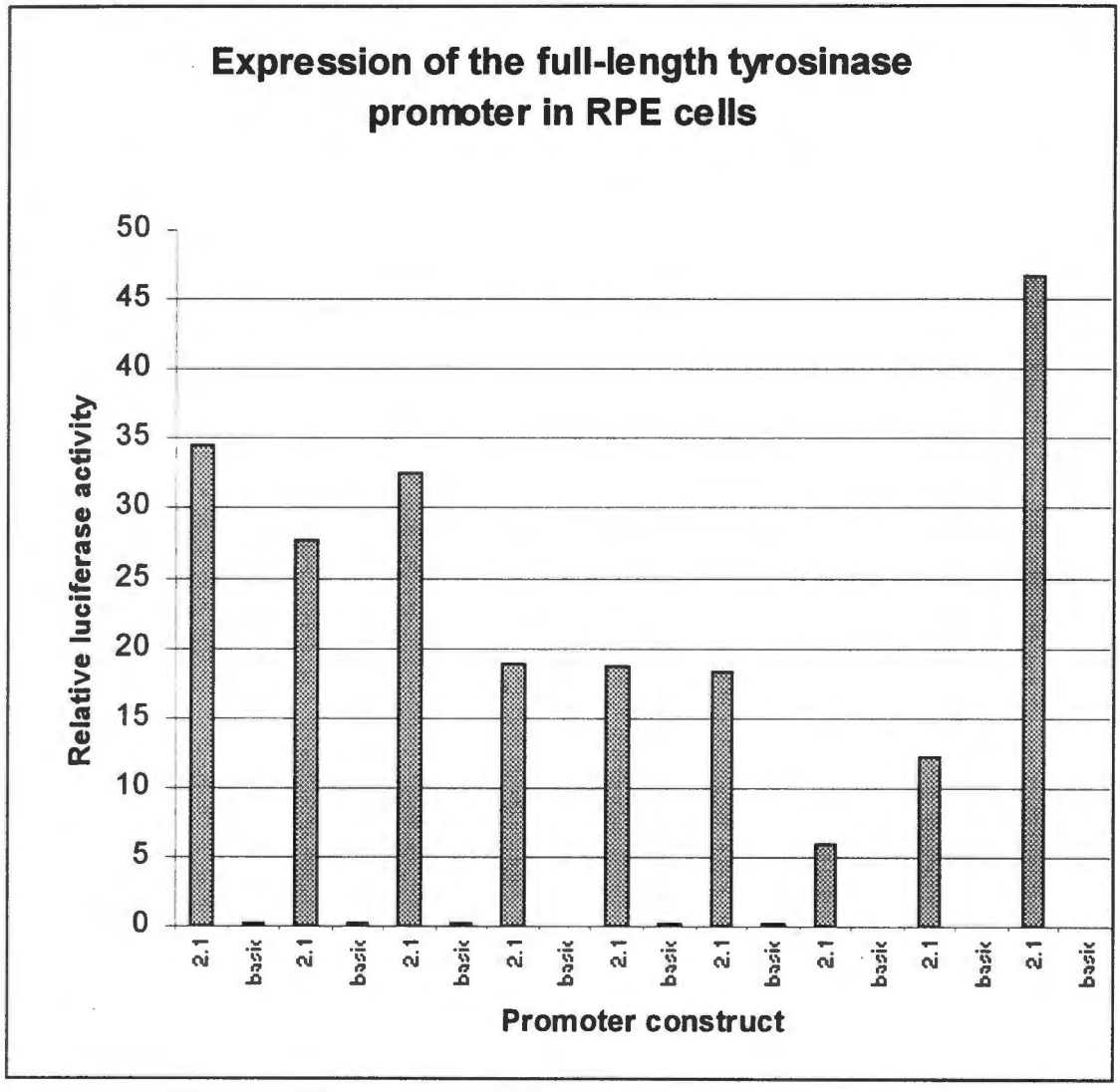
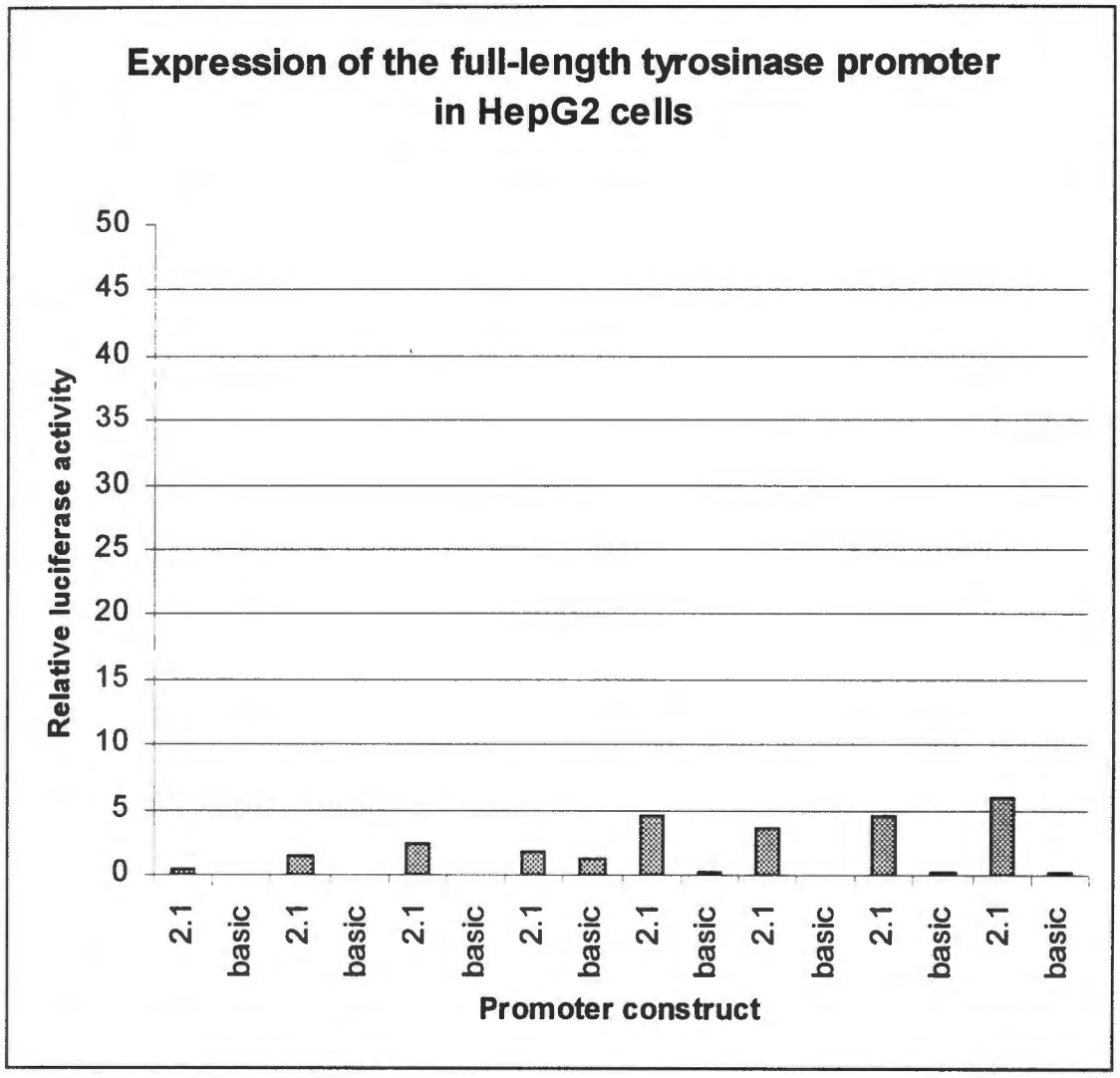


Fig 3: Activity of the full-length chicken tyrosinase promoter construct, Tyr2.1-Luc, in HepG2 cells. The experiment was repeated 8 times. Results were normalised with respect to *Renilla* luciferase activity in the same extract and are expressed as a percentage of the luciferase activity obtained with the positive control vector, pGL2-Luc. The figures 2.1 and basic on the x-axis represent the constructs Tyr2.1-Luc and pGL2-basic, respectively.



REFERENCES

Akiyama, Y., Whitaker, B., Ferderspiel, M., Hughes, S.H., Yamamoto, H., Takeuchi, Y and Brumbaugh, J.: Tissue-specific expression of mouse tyrosinase gene in cultured chicken cells. Exp. Cell Res. 214 (1994) 154-162.

Amae, S., Fuse, N., Yasumoto, K-I., Sato, S., Yajima, I., Yamamoto, H., Udono, T., Durlu, Y.K., Tamai, M., Takahashi, K and Shibahara, S.: Identification of a novel isoform of microphthalmia-associated transcription factor that is enriched in retinal pigment epithelium. Biochem. Biophys. Res. Comm. 247 (1998) 710-715.

Beermann, F., Schedl, A., Ruppert, S., Ganss, R., Schmid, E., Klüppel, M., Hummler, E. and Schütz, G.: The mouse tyrosinase gene: pigment cell-specific gene expression in transgenic mice. *In* From molecular biology to therapeutics. Pharmacol. Skin. Bernanrd, B.A. and Shroot, B., eds. Krager, Basel. 5 (1993) pp. 57-62.

Bennett, D.C., Cooper, P.J. and Hart, I.R.: A line of non-tumorigenic mouse melanocytes, syngeneic with the B16 melanoma and requiring a tumour promoter for growth. Int. J. Cancer 39 (1987) 414-418.

Bentley, N.J., Eisen, T. and Goding, C.R.: Melanocyte-specific expression of the human tyrosinase promoter: activation by the microphthalmia gene product and role of the initiator. Mol. Cell. Biol. 14:12 (1994) 7996-8006.

Bertolotto, C., Busca, R., Abbe, P., Bille, K., Aberdam, E., Ortonne, J-P. and Ballotti, R.: Different *cis*-acting elements are involved in the regulation of Trp1 and Trp2 promoter activities by cyclic AMP: pivotal role of M boxes (GTCATGTGCT) and of microphthalmia. Mol. Cell. Biol. 18 (1998) 694-702.

Boissy, R.E. and Halaban, R.: Establishment of proliferative, pure cultures of pigmented chicken melanocytes from neural tubes. J. Invest. Dermatol. 84 (1985) 158-161.

Carreira, S., Dexter, T.J., Yavuzer, U., Easty, D.J. and Goding, C.R.: Brachyury-related transcription factor Tbx2 and repression of the melanocyte-specific Trp1 promoter. Mol. Cell. Biol. 18:9 (1998) 5099-5108.

Chen, C. and Okayama, H.: High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. 7:8 (1987) 2745-2752.

Chiu, E., Lamoreux, M.L. and Orlow, S.J.: Postnatal ocular expression of tyrosinase and related proteins: disruption by the *pink-eyed unstable* (p^{un}) mutation. Exp. Eye. Res. 57 (1993) 301-305.

Davis, L.G., Dibner, M.D. and Battey, J.F.: Basic methods in molecular biology. Elsevier Science Publishing Co., Inc., NY, 1986.

Del Marmol, V. and Beermann, F.: Tyrosinase and related proteins in mammalian pigmentation. FEBS lett. 381 (1996) 165-168.

Edlund, T., Walker, M.D., Barr, P.J. and Rutter, W.J.: Cell-specific expression of the rat insulin gene: evidence for role of two distinct 5' flanking elements. Science 230 (1985) 912-916.

Eguchi, G. and Okada, T.S.: Differentiation of lens tissue from the progeny of chick retinal pigment cells cultured *in vitro*: a demonstration of a switch of cell types in clonal cell culture. Proc. Natl. Acad. Sci. USA. 70 (1973) 1495-1499.

Eisen, T., Easty, D.J., Bennett, D.C. and Goding, C.R.: The POU domain transcription factor Brn-2: elevated expression in malignant melanoma and regulation of melanocyte-specific gene expression. Oncogene 11 (1995) 2157-2164.

Fauquet, M., Stehelin, D. and Saule, S.: myc products induce the expression of catecholaminergic traits in quail neural crest-derived cells. Proc. Natl. Acad. Sci. USA. 87 (1990) 1546-1550.

Ferguson, C.A. and Kidson, S.H.: Characterstic sequences in the promoter region of the chicken tyrosinase-encoding gene. Gene 169 (1996) 191-195.

Ferguson, C.A. and Kidson, S.H.: The regulation of tyrosinase gene transcription. Pigment Cell Res. 10 (1997) 127-138.

Fuse, N., Yasumoto, K-I., Suzuki, H., Takahashi, K. and Shibahara, S.: Identification of a melanocyte-type promoter of the microphthalmia-associated transcription factor gene. Biochem. Biophys. Res. Com. 219 (1996) 702-707.

Ganss, R., Schütz, G. and Beermann, F.: The mouse tyrosinase gene: promoter modulation by positive and negative regulatory elements. J. Biol. Chem. 269:47 (1994) 29808-29816.

Ganss, R., Montoliu, L., Monaghan, A.P. and Schütz, G.: A cell-specific enhancer far upstream of the mouse tyrosinase gene confers high level and copy number-related expression in transgenic mice. EMBO J. 13 (1994) 3083-3093.

Gilbert, S.F.: Developmental biology. Fourth edition. Sinauer Associates Inc., Massachusetts, 1994.

Gorman, C.M., Moffat, L.F. and Howard, B.H.: Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. 2 (1982) 1044-1051.

Grosveld, F., Blom van Assendelft, G., Greaves, D.R. and Kollias, G.: Position independent, high level expression of the human β-globin gene in transgenic mice. Cell 51 (1987) 975-985.

Herbomel, P., Bourachot, B. and Yaniv, M.: Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. Cell 39 (1984) 653-662.

Hjelmeland, L.M.: Senescence of the retinal pigment epithelium. Invest. Ophthalmol. Vis. Sci. 40:1 (1999) 1-2.

Hill, H.Z., Li, W., Xin, P. and Mitchell, D.L.: Melanin: A two edged sword? Pigment Cell Res. 10 (1997) 158-161.

Itoh, Y. and Eguchi, G.: *In vitro* analysis of cellular metaplasia from pigmented epithelial cells to lens phenotypes: a unique model system for studying cellular and molecular mechanisms of 'transdifferentiation'. Dev. Biol. 115 (1986) 353-362.

Jackson, I.J., Chambers, D.M., Budd, P.S. and Johnson, R.: The tyrosinase-related protein-1 gene has a structure and promoter sequence very different from tyrosinase. Nucl. Acids Res. 19:14 (1991) 3799-3804.

Jackson, I.J. and Raymond, S.: Manifestations of *microphthalmia*. Nature Genet. 8 (1994) 209-210.

Kennedy, B.N., Goldflam, S., Chang, M.A., Campochiaro, P., Davis, A.A., Zack, D.J. and Crabb, J.W.: Transcriptional regulation of cellular retinaldehyde-binding protein in the retinal pigment epithelium: a role for the photoreceptor consensus element. J. Biol. Chem. 273:10 (1998) 5591-5598.

Kikuchi, H., Miura, H., Yamamoto, H., Takeuchi, T., Dei, T. and Watanabe, M.: Characteristic sequences in the upstream region of the human tyrosinase gene. Biochim. Biophys. Acta 1009 (1989) 283-286.

Kingston, R.E.: Transfection of DNA into eukaryotic cells: calcium phosphate transfection. *In* Current Protocols in Molecular Biology. Ausubel, F.M., Brent, F., Kingston, R.E., Moore, D.D., Smith, J.A., Seidman, J.G. and Struhl, K., eds. Greene, New York, 1987, pp. 9.1.1-9.1.4.

Klüppel, M., Beermann, F., Ruppert, S., Schmid, E., Hummler, E. and Schütz, G.: The mouse tyrosinase promoter is sufficient for expression in melanocytes and the pigmented epithelium of the retina. Proc. Natl. Acad. Sci. USA. 88 (1991) 3777-3781.

Kreitner, P.C.: Linkage studies in a new black-eyed white mutation in the house mouse (not *W*). J. Hered. 48 (1957) 300-304.

Kwon, B.S., Haq, A.K., Pomerantz, S.H. and Halaban, R.: Isolation and sequence of a cDNA clone for human tyrosinase that maps at the mouse c-albino locus. Proc. Natl. Acad. Sci. USA. 84 (1987) 7473-7477.

Lister, J.A., Robertson, C.P., Lepage, T., Johnson, S.L. and Raible, D.W.: *nacre* encodes a zebrafish micropthalmia-related protein that regulates neural-crest-derived pigment cell fate. Development 126 (1999) 3757-3767.

Lowings, P., Yavuzer, U. and Goding, C.R.: Positive and negative elements regulate a melanocyte-specific promoter. Mol. Cell. Biol. 12:8 (1992) 3653-3662.

Matheny, A.P. and Dolan, A.B.: Changes in eye colour during early childhood: sex and genetic differences. Ann. Hum. Biol. 2:2 (1975) 191-196.

Montoliu, L., Umland, T. and Schütz, G.: A locus control region at –12kb of the tyrosinase gene. EMBO J. 15:22 (1996) 6026-6034.

Moore, K.J.: Insight into the *microphthalmia* gene. TIG. 11:11 (1995) 442-448.

Moore, K.L.: The developing human: clinically oriented embryology. Second edition. W.B. Saunders Company, Philadelphia, 1977.

Morrison, R., Mason, K. and Frost-Mason, S.: A cladistic analysis of the evolutionary relationships of the members of the tyrosinase gene family using sequence data. Pigment Cell Res. 7 (1994) 388-393.

Nakayama, A., Nguyen, M-T. T., Chen, C.C., Opdecamp, K., Hodgkinson, C.A and Arnheiter, H.: Mutations in *micropthalmia*, the mouse homolog of the human deafness gene *MITF*, affect neuroepithelial and neural crest-derived melanocytes differently. Mech. Devel. 70 (1998) 155-166.

Nicoletti, A., Kawase, K. and Thompson, D.A.: Promoter analysis of *RPE65*, the gene encoding a 61-kDa retinal pigment epithelium-specific protein. Invest. Ophth. Vis. Sci. 39:3 (1998) 637-644.

Park, H-Y., Russakovsky, V., Ohno, S and Gilchrest, B.A.: The beta isoform of protein kinase C stimulates human melanogenesis by activating tyrosinase in pigment cells. J. Biol. Chem. 268 (1993) 11742-11749.

Pierro, L.J.: Effects of the *light* mutation of mouse coat colour on eye pigmentation. J. Exp. Zool. 153 (1963) 81-87.

Ponnazhagen, S. and Kwon, B.S.: A *cis*-acting element involved in mouse tyrosinase gene expression and partial purification of its binding protein. Pigment Cell Res. 5 (1992) 155-161.

Porter, S., Larue, L. and Mintz, B.: Mosaicism of tyrosinase-locus transcription and chromatin structure in dark vs. light melanocyte clones of homozygous chinchilla-mottled mice. Dev. Genet. 12 (1991) 393-402.

Porter, S.D. and Meyer, C.J.: A distal tyrosinase upstream element stimulates gene expression in neural-crest-derived melanocytes of transgenic Mice: position-independent and mosaic expression. Development 120 (1994) 2103-2111.

Porter, S.D., Hu, J. and Gilks, C.B.: Distal upstream tyrosinase S/MAR-containing sequence has regulatory properties specific to subsets of melanocytes. Dev. Genet. 25 (1999) 40-48.

Promega Corporation, Protocols and Applications Guide, 3rd Edition, 1996.

Prota, G.: Pigment cell research: What directions? Pigment Cell Res. 10 (1997) 5-11.

Raymond, S.M. and Jackson, I.J.: The retinal pigment epithelium is required for development and maintenance of the mouse neural retina. Current Biology 5 (1995) 1286-1295.

Sambrook, J., Fritsch, E.F. and Maniatis, T.: Molecular cloning. A laboratory manual. Second edition. Cold Spring Harbour Laboratory Press, New York, 1989.

Schmidt, A., Tief, K., Yavuzer, U. and Beermann, F.: Ectopic expression of *ret* results in microphthalmia and tumors in the retinal pigment epithelium. Int. J. Cancer 80 (1999) 600-605.

Schraermeyer, U. and Heimann, K.: Current understanding on the role of retinal pigment epithelium and its pigmentation. Pigment Cell Res. 12 (1999) 219-236.

Shibata, K., Muraosa, Y., Tomita, Y., Tagami, H. and Shibahara, S.: The identification of a *cis*-acting element that enhances the pigment cell-specific expression of the human tyrosinase gene. J. Biol. Chem. 267 (1992) 20548-20588.

Sidman, R.L., Kosaras, B. and Tang, M.: Pigment epithelial and retinal phenotypes in the vitiligo, *mi*^{vit}, mutant mouse. Inv. Ophth. Vis. Sci. 37:6 (1996) 1097-1115.

Smale, S.: Transcription initiation from TATA-less promoters within eukaryotic protein-coding genes. Biochim. Biophys. Acta 1351 (1997) 73-88.

Smith, S.B., Zhou, B.K. and Orlow, S.J.: Expression of tyrosinase and the tyrosinase related proteins in the *Mitf*^{vit} (vitiligo) mouse eye: implications for the function of the microphthalmia transcription factor (Mitf). Exp. Eye Res. 66 (1998) 403-410.

Sturm, R.A., O'Sullivan, B.J., Box, N.F., Smith, A.G., Smit, S.E., Puttick, E.R.J., Parsons, P.G. and Dunn, I.S.: Chromosomal structure of the human Tyrp1 and Tyrp2 loci and comparison of the tyrosinase-related protein gene family. Genomics 29 (1995) 24-34.

Takeuchi, S., Takeuchi, T. and Yamamoto, H.: A possible mechanism for feedback regulation of the mouse tyrosinase gene by its 3' non-coding RNA fragments. Pigment Cell Res. 13 (2000) 109-115.

Tief, K., Schmidt, A. and Beermann, F.: New evidence for the presence of tyrosinase in substania nigra, forebrain and midbrain. Mol. Brain. Res. 53 (1998) 307-310.

Udono, T., Yasumoto, K-I., Takeda, K., Amae, S., Watanabe, K-I., Saito, H., Fuse, N., Tachibana, M., Takahashi, K., Tamai, M. and Shibahara, S.: Structural organisation of the human microphthalmia-associated transcription factor gene containing four alternative promoters. Biochim. Biophys. Acta 1491 (2000) 205-219.

Watanabe, A., Takeda, K., Ploplis, B. and Tachibana, M.: Epistatic relationship between Waardenburg syndrome genes *MITF* and *PAX3*. Nature Genet. 18 (1998) 283-286.

Wolpert, L., Beddington, R., Brockes, J., Jessel, T., Lawrence, P. and Meyerowitz, T.: Principles of development. Current Biology Publications, London and Oxford University Press, Oxford, 1998.

Wu, M., Rinchik, E.M., Wilkinson, E. and Johnson, D.K.: Inherited somatic mosaicism caused by an intracisternal A particle insertion in the mouse tyrosinase gene. Proc. Natl. Acad. Sci. USA. 94 (1997) 890-894.

Yamamoto, H., Takeuchi, S., Kudo, Y., Sato, C. and Takeuchi, T.: Melanin production in cultured albino melanocytes transfected with mouse tyrosinase cDNA. Jpn. J. Genet. 64 (1989) 121-135.

Yamamoto, H., Kudo, T., Masuko, N., Miura, H., Sato, S., Tanaka, M., Tanaka, S., Takeuchi, S., Shibahara, S. and Takeuchi, T.: Phylogeny of regulatory regions of vertebrate tyrosinase genes. Pigment Cell Res. 5 (1992) 284-294.

Yasumoto, K-I., Yokoyama, K., Shibata, K., Tomita, Y and Shibahara, S.: Micropthalmia-associated transcription factor of the human tyrosinase gene. Mol. Cell. Biol. 14:12 (1994) 8058-8070.

Yasumoto, K-I., Mahalingam, H., Suzuki, H., Yoshizawa, M., Yokoyama, K. and Shibahara, S.: Transcriptional activation of the melanocyte-specific genes by the human homolog of the mouse *micropthalmia* protein. J. Biochem. 118 (1995) 874-881.

Yasumoto, K-I., Yokoyama, K., Takahashi, K., Tomita, Y. and Shibahara, S.: Functional analysis of microphthalmia-associated transcription factor in pigment cell-specific transcription of the human tyrosinase family genes. J. Biol. Chem. 272:1 (1997) 503-509.

Yasumoto, K-I., Amae, S., Udono, T., Fuse, N., Takeda, K. and Shibahara, S.: A big gene linked to small eyes encodes multiple mitf isoforms: many promoters make light work. Pigment Cell Res. 11 (1998) 329-336.

Zhao, S., Rizzolo, L.J. and Barnstable, C.J.: Differentiation and transdifferentiation of the retinal pigment epithelium. Int. Rev. Cytol. 171 (1997) 225-266.

Zhou, B-K., Boissy, R.E., Pifko-Hurst, S., Moran, D. and Orlow, S.J.: Lysosome-associated membrane protein-1 (LAMP-1) is the melanocyte vesicular membrane glycoprotein band II. J. Invest. Dermatol. 100 (1993) 110-114.